PROCESS FOR PROPAGATION AND/OR SELECTION OF PLANT MATERIAL

Publication number: WO9723126
Publication date: 1997-07-03
Inventor: EDWARDS GLYN ALYN (GB); PURSE JOHN GILBERT (GB)
Applicant: SHELL INT RESEARCH (NL); EDWARDS GLYN ALYN (GB); PURSE JOHN GILBERT (GB)
Classification:
- international: ***A01H1/00; A01H1/04; A01H4/00;*** **A01H1/00; A01H1/04; A01H4/00;** (IPC1-7): A01H1/04
- European: A01H1/04; A01H4/00D
Application number: WO1996EP05746 19961216
Priority number(s): EP19950309401 19951222; EP19960306008 19960816

Also published as:

- WO9723126 (A3)
- OA10799 (A)
- PT877546T (E)
- ES2163049T (T3)
- AU721423B (B2)

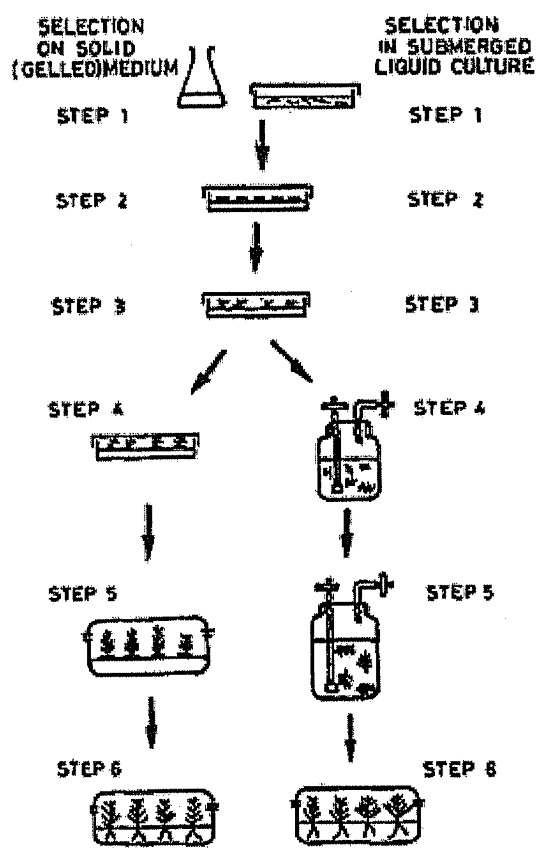
Cited documents:

- US4569914
- XP002031755
- JP8089113
- XP002031756
- JP7203790
- more >>

Report a data error here

Abstract of **WO9723126**

A process for the micropropagation of shoots, rooted shoots or seedlings of a woody plant, which comprises cultivating the shoots, rooted shoots or seedlings in an oxygenated liquid culture medium, the shoots, rooted shoots or seedlings being submerged in the culture medium. They may move freely, for example, tumbling in the medium, or their movement may be restricted. The resulting plant material is obtained in high yields and has high quality. When genetically modified shoots, rooted shoots and seedlings have a selectable property, for example, resistance to a herbicide or antibiotic, they may be selected by submerged cultivation in an oxygenated liquid culture medium that comprises means for the selection, for example, the herbicide or antibiotic.



PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A01H

A2

(11) International Publication Number: WO 97/23126

(43) International Publication Date: 3 July 1997 (03.07.97)

(21) International Application Number: PCT/EP96/05746

(22) International Filing Date: 16 December 1996 (16.12.96)

(30) Priority Data:
95309401.8 22 December 1995 (22.12.95) EP
(34) Countries for which the regional or international application was filed: GB et al.
96306008.2 16 August 1996 (16.08.96) EP
(34) Countries for which the regional or international application was filed: GB et al.

(71) Applicant *(for all designated States except US)*: SHELL INTERNATIONALE RESEARCH MAATSCHAPPIJ B.V. [NL/NL]; Carel van Bylandtlaan 30, NL-2596 HR The Hague (NL).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: EDWARDS, Glyn, Alyn [GB/GB]; 25 Blenheim Avenue, Faversham, Kent ME13 8NW (GB). PURSE, John, Gilbert [GB/GB]; 2 Champion Court Cottages, Newnham, Sittingbourne, Kent ME9 0JX (GB).

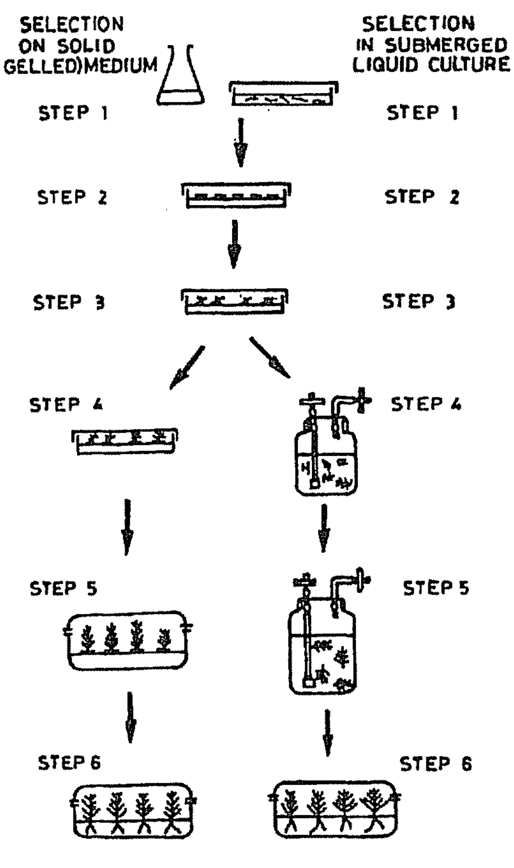
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: PROCESS FOR PROPAGATION AND/OR SELECTION OF PLANT MATERIAL

(57) Abstract

A process for the micropropagation of shoots, rooted shoots or seedlings of a woody plant, which comprises cultivating the shoots, rooted shoots or seedlings in an oxygenated liquid culture medium, the shoots, rooted shoots or seedlings being submerged in the culture medium. They may move freely, for example, tumbling in the medium, or their movement may be restricted. The resulting plant material is obtained in high yields and has high quality. When genetically modified shoots, rooted shoots and seedlings have a selectable property, for example, resistance to a herbicide or antibiotic, they may be selected by submerged cultivation in an oxygenated liquid culture medium that comprises means for the selection, for example, the herbicide or antibiotic.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgystan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic			SE	Sweden
CG	Congo	KR	Republic of Korea	SG	Singapore
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LR	Liberia	SZ	Swaziland
CS	Czechoslovakia	LT	Lithuania	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	LV	Latvia	TJ	Tajikistan
DK	Denmark	MC	Monaco	TT	Trinidad and Tobago
EE	Estonia	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	UG	Uganda
FI	Finland	ML	Mali	US	United States of America
FR	France	MN	Mongolia	UZ	Uzbekistan
GA	Gabon	MR	Mauritania	VN	Viet Nam

PROCESS FOR PROPAGATION AND/OR SELECTION OF PLANT MATERIAL

The present invention relates to a process for the vegetative propagation of plant material and/or for the selection of genetically modified plant material.

In vitro vegetative propagation (micropropagation) techniques are widely used for commercial-scale production of plants. Those techniques have the potential advantage over other methods of vegetative propagation, for example, propagation via cuttings, that a larger number of plants can be produced in a given time period. Furthermore, the techniques are useful for propagating genetically manipulated plant material. In the case of some plant genotypes, micropropagation techniques may be the only method available for vegetative propagation. However, although widely used in horticulture, commercial use of micropropagation techniques for the propagation of woody plants, for example, trees, is not widespread.

The vast majority of micropropagation techniques for vegetative propagation of woody plants use gelled growth media or use other physical means to support the plant material in or on a liquid growth medium. (Gelled media are often called "solid" whether they are in fact solid or semi-solid. Unless specified otherwise, the terms "solid media" and "semi-solid media" as used herein include both solid and semi-solid forms of gelled media.) However, micropropagation techniques that employ solid media or physical supports have the disadvantage that they are generally labour intensive and expensive. In addition, micropropagation techniques for woody plants often suffer from the disadvantage that it is difficult

to produce uniform products. The reason for this is that it is often difficult to induce existing meristems, for example, axillary buds, to develop uniformly. That may be due to the meristems being quiescent or dormant and/or due to non-uniform elongation and development once growth has started. For commercial production, uniformity of product is extremely important. Any developmental heterogeneity of shoots set for rooting is often amplified during subsequent development of the plants.

There are therefore clear incentives to develop alternative micropropagation techniques for woody plants that are more efficient, more cost-effective and capable of producing a more uniform product.

It has been proposed to use submerged liquid media for micro-propagation as there are a number of potential advantages over solid media, including a greater ability to automate and hence to reduce expenses. Submerged culture of plant tissues has been employed extensively for cell culture i.e. growth of single cells, small groups of undifferentiated cells and embryogenic tissues or meristematic tissues. However, attempts to use liquid media to propagate shoots and plantlets has been hampered by a number of problems, and it appears that the technique has had only very limited application, to certain specific plants.

Hyperhydricity of shoots is a problem often encountered in liquid micropropagation systems, see for example, Aitken-Christie et al 1994 and George E F 1993/1996.

Hyperhydricity was previously termed vitrification (water-logging), but that is not technically correct. Hyperhydricity refers to the condition of in vitro cultured material that has an abnormal morphological appearance and physiological function. Susceptibility to hyperhydricity is not the same in all plants. Typical

symptoms are shoots with shorter inter-nodes, brittle, curled or translucent leaves, abnormal anatomy including large intercellular spaces, reduced vascular system and surface wax; poorly developed stomata and chloroplasts; and altered biochemical characteristics including reduced lignin and cellulose and altered enzyme activities.

Virtually all plants cultured in vitro, even when grown on semi-solid media, show to some extent some of the symptoms associated with hyperhydricity. However, visual symptoms are irrelevant if those symptoms are mild and can be reversed easily. In such cases, the mild symptoms may not adversely affect plant propagation. The material under propagation can be recovered i.e. rooted, and the subsequent performance and hence value of the propagules is not affected adversely.

Hyperhydricity may cause problems in plant propagation when extreme expression of the characteristic symptoms described above is encountered. Hyperhydric shoots that show extreme expression of the characteristic symptoms under in vitro propagation conditions generally become difficult to propagate and may die whilst still in culture. They do not normally produce adventitious roots or root only poorly. Hyperhydritic shoots rarely survive transfer from an in vitro environment, for example, to the greenhouse. Such shoots are therefore generally unsuitable for rooting and/or subsequent propagation.

Hyperhydricity is most often encountered in the propagation of woody species. In woody plants, hyperhydricity of shoots and plantlets is a widespread problem and may be severe. Indeed, many woody plants are prone to severe hyperhydricity even when grown on solid or semi-solid growth media.

Severe hyperhydricity is an extremely common phenomenon in liquid micropropagation systems. For that reason, liquid systems are generally avoided by the use

of solid or semi-solid supports, for example, gelled media. In most of the cases where a liquid system is used, careful precautions are taken to avoid complete submersion of the plant material in the liquid culture medium.

A number of propagation techniques have been developed in which shoots or plantlets are floated on a liquid medium or grown in non-submerged (shallow) liquid media systems or in systems in which they are periodically flooded with culture medium. Gupta et al (Plant Sci Lett 20: 195-201, 1981) describe a method for multiplying and rooting seedlings and mature clones of Eucalyptus citriodora using protocols that have both solid and semi-submerged liquid culture media stages. However, such techniques have only limited advantages compared to techniques employing solid or semi-solid media.

Attempts have been made to reduce hyperhydricity in liquid culture by using growth retardants such as paclobutrazol and ancymidol, which reduce the growth of the worst affected tissue, i.e. leaves. However, the use of growth retardants has been successful only with species such as Gladiolus or Nerine that are propagated in vitro via tubers or corms.

The present invention provides a process for the micro-propagation of shoots, rooted shoots or seedlings of a woody plant, which comprises cultivating the shoots, rooted shoots or seedlings in an oxygenated, for example, aerated liquid culture medium, the shoots, rooted shoots or seedlings being submerged in the liquid medium. The medium is generally agitated or otherwise moving.

The shoots, rooted shoots or seedlings may be allowed to move in the liquid medium, for example, they may be allowed to move freely, for example, to tumble, for example, tumble freely, in the liquid medium.

Alternatively, their movement may be restricted or otherwise impeded.

The process of the invention is simple, inexpensive in itself, reduces labour costs and gives excellent quality material that is suitable for large-scale commercial use, as shown in Figure 1 of the accompanying drawings.

Figure 1 shows four shoot masses:

1: Typical micropropagated Eucalyptus grandis shoot used as inoculum.

2: Typical Eucalyptus grandis shoot mass produced after five weeks cultivation on solid micropropagation medium (KM medium).

3a,3b: Two typical Eucalyptus grandis shoot masses harvested after 27 days submerged liquid culture in liquid KM medium according to the present invention.

In the process of the present invention, the shoots, rooted shoots or seedlings are cultured under conditions such that they are completely immersed in the liquid medium. The shoots, rooted shoots or seedlings may be allowed to move in the liquid medium, for example, they may be allowed to move freely. For example, the shoots, rooted shoots or seedlings may tumble, for example, tumble freely, in the liquid medium. The tumbling may be vigorous and may be substantially continuous. Alternatively, the movement of the shoots, rooted shoots or seedlings may be restricted or otherwise impeded. For example, the shoots, rooted shoots or seedlings may be restrained, for example, they may be held by perforated restraining means, for example, in a perforated container, for example, a cage or bag, within the plant growth vessel or within a separate section of the vessel that is in contact with the liquid medium.

The liquid medium in which the plant material is cultured must comprise sufficient oxygen to support the

metabolism of the plant material. It is generally necessary to provide oxygen, usually in the form of air, and/or to illuminate the system such that oxygen is produced by photosynthesis.

The medium may be agitated by mechanical means, for example, by means of a mechanical device, for example, a paddle or a stirrer, for example, a magnetic stirrer, or the vessel containing the medium may be agitated, for example, shaken, vibrated or rotated. The means used for agitation, for example, shaking or stirring, may oxygenate the medium sufficiently to support the metabolism of the plant material. If not, oxygen, generally in the form of air, may be provided and/or the system may be illuminated such that oxygen is produced by photosynthesis.

The production of oxygen by photosynthesis may totally or in part provide the oxygen required by the culture. The light required for the production of oxygen by photosynthesis will generally cause movement of the liquid medium by convection.

The liquid medium may be both agitated and oxygenated by passing oxygen or, more usually, air, through the medium. Air circulation techniques, sometimes called "airlift" techniques, are particularly useful for providing simultaneous oxygenation and agitation in the process of the present invention. Liquid medium in an appropriate vessel, for example, a fermentation vessel, for example, a flask, bottle, tank or column may be circulated and oxygenated by the introduction of air through, for example, a gas diffuser. Such vessels are often called "air-lift fermenters". The volume of air and the rate of introduction can easily be adjusted to give the desired degree of agitation to the liquid medium and hence to the plant material when it is allowed to move freely.

When the plant material is allowed to move freely, agitation of the medium by passing air or another oxygen-containing gas through the medium may be preferable to agitation by mechanical means, for example, stirring, because the shearing forces on the plant material may be lower. However, the shearing forces on the plant material in shaken or stirred systems of the present invention may by reduced by restricting the movement of the plant material, for example, as described in more detail below. In a system where air or another gas is passed through the medium it may also be desirable to restrict the movement of the plant material, so the liquid medium is agitated but the plant material is restrained.

If the passage of the gas is not sufficient to achieve the desired agitation, additional agitation means may be provided, for example, the plant growth vessel may be also be shaken or the contents may be stirred. The vessel may be illuminated to provide further oxygen by photosynthesis.

It should be noted that after inoculation in the liquid medium the shoots, rooted shoots or seedlings may not become wetted readily because air bubbles may bind to the surfaces. If so, the shoots, rooted shoots or seedlings may float at or near the surface of the medium. A surfactant may be included in the culture medium to assist wetting. Even in the absence of surfactant, however, the surfaces of the shoots, rooted shoots or seedlings will generally become thoroughly wetted within a few days. In an air-lift system, if there is sufficient agitation and they are not restricted, they will generally tumble freely.

As indicated above, the shoots, rooted shoots or seedlings that are propagated according to the submerged liquid culture process of the present invention may be

allowed to move freely in the liquid culture medium or their movement may be restricted or otherwise impeded.

The plant material may be physically restrained in a container within the plant growth vessel or the vessel may be divided into sections, one or more sections containing plant material. The restraining or dividing means should allow passage of the sufficient liquid medium to allow adequate oxygenation of the plant material. Perforated or mesh materials may be used to construct the restraining means, for example, there may be used a perforated metal or plastics material, a wire mesh or a fabric, for example, muslin. Alternatively, fresh oxygen-containing liquid medium may be passed through a plant growth vessel containing the plant material, either continuously or periodically, the plant material being submerged at all times.

The movement of the plant material may be restricted in any the liquid culture system of the present invention, including those where oxygenation is achieved by shaking or stirring the liquid medium, by passing air or another oxygen-containing gas through the liquid medium, by photosynthesis, or by any combination of thereof. Restricting the movement of the plant material may have the advantage of reducing the shearing forces on the material. In the case of stirred cultures, it is particularly preferable to restrain the plant material because it may be damaged by the stirrer if it is allowed to move freely.

The liquid medium used for the propagation may be any medium that is suitable for the propagation of the chosen shoots, rooted shoots or seedlings. It should generally include sources of carbon and of nitrogen, organic and inorganic salts as required, and appropriate phytohormones and/or plant growth regulators. Suitable growth media are known, and an approp-riate medium may be chosen

for the particular woody plant to be propagated. Known solid media may be modified by the omission of the gelling or other solidification agent to give a liquid medium. It may also be appropriate to modify levels of one or more of the components of a known medium for use according to the present invention, for example, it may be possible to use reduced levels of phytohormones and/or plant growth regulators compared with levels used in corresponding solid media. The optimum level of any particular component or combinations of components may be determined by conventional methods.

Appropriate culture conditions should be used, for example, with regard to temperature and light. It may be preferable to illuminate the culture to provide endogenous oxygenation to supplement or even, in some cases, to replace exogenous oxygenation. If the culture undergoes autotrophic growth under light, the carbohydrate source in the medium may be omitted or reduced. In such cases, the supply of air or another oxygen-containing gas may be suppplemented with carbon dioxide. Alternatively, if sufficient oxygen is provided to support the metabolism of the plant material, cultivation may be carried out entirely or partially in the dark.

Shoots, rooted shoots and seedlings produced under cultivation in the dark may be elongated in comparison with those produced under illumination. Elongation may be advantageous for subsequent manipulation, for example, for dissection of the shoot or seedling. Furthermore, elongated shoots of some plants, for example, E. grandis and hybrids thereof, often root more easily than short shoots. Accordingly, the liquid culture system of the present invention enables manipulation of lighting conditions for the manipulation of shoot quality, which is important in commercial micropropagation systems.

Cultivation may be continued until the desired increase in biomass and in multiplication rates is achieved. Shoots may be removed from the liquid culture medium before or after rooting has initiated or has taken place and then transferred to a solid rooting medium for root initiation and/or development. The rooting medium used may contain activated charcoal, if desired. Alternatively, initiation of rooting and/or subsequent root development may be performed in vivo using suitable organic and/or inorganic substrates, for example, vermiculite, compost, soil or peat.

Shoots, rooted shoots (plantlets) or seedlings used for inoculation may be obtained by micropropagation, or germinated and/or grown in a growth chamber or cabinet, greenhouse or outdoors. The shoots, rooted shoots or seedlings may be obtained from a cultivar, clone or seed, especially from genetically valuable cultivars, clones or seed, or from genetically manipulated plant material. Shoots used for inoculation preferably have one or more nodes, for example, from two to four nodes. The shoot tip (apical meristem) may be present, or may be removed before inoculation. Removal of the apical meristem may result in a more uniform product.

Microbial contamination is a potential problem in liquid culture systems in general. In the present case, it may be a particular problem when the starting material has been obtained from plants grown under non-sterile conditions, for example, in a glasshouse or outdoors, even though surface sterilisation or disinfection is carried out according to conventional methods. Even when the starting material has been produced under sterile conditions there may be microbial contamination from other sources.

Accordingly, the liquid culture medium employed in the process of the present invention preferably comprises

an antibiotic, for example, augmentin. However, the fact that the shoot, rooted shoot or seedling masses are totally immersed in the liquid medium should result in more efficient penetration of antibiotic into the tissues of the shoot masses than occurs on solid media, and hence the effect of microbial contamination, should it occur, is reduced.

This is particularly important for micropropagation according to the process of the present invention of genetically manipulated plant material obtained using Agrobacterium-mediated transfer. Such material may present particular problems for subsequent micropropagation because of the inevitable contamination with the Agrobacteria themselves. Such problems are minimised in the process of the present invention.

Nevertheless, precautions are preferably taken to minimise the potential for contamination by exogenous microorganisms, for example, any supply of air or other gas used for oxygenation is preferably filtered, irradiated, chemically or otherwise treated to remove microorganisms; joints and connections are preferably sealed, and apparatus is preferably sterilised before use, for example, by autoclaving. Tissue culture grade materials, for example, tubing, are preferably used.

The process of the present invention is applicable to woody plants, that is to say, perennial plants that exhibit secondary growth (secondary thickening) of roots and/or aerial stems, which is the result of the formation of wood. Wood is secondary xylem, and is composed of one or more of the following: tracheids, vessels, fibres and rays. Woody plants include forest trees, other trees, shrubs and bushes.

Examples of woody plants that may be micropropagated by the process of the present invention include, but are not limited to, gymnosperms and dicotyledenous

angiosperms, for example, as used for wood pulp, for fuel or for timber, for example, Eucalyptus, Pinus, Picea, Acacia, Populus, Betula, Tectona and tropical hardwoods; trees, shrubs and bushes that produce fruit or nuts, for example, apple, citrus, peach, olive, walnut and almond trees, coffee bushes, blackcurrant bushes, and raspberry canes; trees, shrubs and bushes from which other commercially useful products can be obtained, for example, rubber trees and trees and shrubs that produce pharmaceutically useful substances or precursors for pharmaceutically useful substances, for example, yew trees; and ornamental trees and shrubs, for example, trees and shrubs having ornamental flowers, foliage or bark.

Woody plants that appear to perform particularly well in the liquid culture system of the present invention are sclerophyllous species. The definition of sclerophyll is "thick, leathery leaf". This includes true leaves, as in the case of eucalypts and phyllodes as in the case of some Acacias. Species considered as sclerophyllous are generally evergreen and the sclerophyllous habit is generally associated with poor nutrient availability and often with drought tolerance. Examples of sclerophyllous genera are Rhododendron, Azalea and Kalmia (Ericaceae); Olea (Oleaceae); many Australian Acacias (Fabaceae); and eucalypts (Myrtaceae).

However, it is not only sclerophyllous species that perform well in the liquid culture system of the present invention. Malus (apple), Pyrus, Prunus and Rosa (Rosaceae), Forsythia and Syringa (Oleaceae) are further examples of woody plant genera that may be propagated using the liquid culture system of the present invention.

As indicated above, eucalyptus is an example of a sclerophyllous woody plant that may be propagated according to the present invention. The sub-genus

Eucalyptus symphyomyrtus contains many commercially useful species, for example, E. grandis, E. globulus, E. nitens, E. dunnii, E. saligna, E. camaldulensis, E. urophylla and hybrids thereof. Further commercially important Eucalyptus species include E. regnans, E. citriodora, E. fraxinoides and hybrids thereof.

According to a particularly preferred embodiment of the process of the present invention, shoots, rooted shoots or seedlings are propagated in a submerged liquid culture that is oxygenated and agitated by means of circulating air such that the shoots or plants tumble, preferably freely. In such a system, which is often called an "air-lift" system, compressed air (or other suitable gas) is fed into a plant growth vessel containing a liquid medium and the shoots, rooted shoots or seedlings. The air or other gas supplied is preferably humidified before it enters the plant growth vessel, for example, by passage through water, especially distilled water.

According to further preferred embodiments of the invention, the shoots, rooted shoots or seedlings are propagated in a vessel that is shaken or stirred. It is particularly preferred to restrict the movement of the plant material, for example, in perforated container, for example, a cage or bag as described above. It may also be advantageous to restrict the movement of the plant material in a culture system of the invention where air is passed through the liquid medium.

As indicated above, it is preferable to ensure that all apparatus and all other materials used are sterile to minimise microbial contamination. Apparatus is preferably sterilised before use by autoclaving, and media are sterilied by autoclaving or filtration, where possible, and tissue culture grade materials are preferably used. All joints in the apparatus should be

carefully sealed.

Where air or another gas is supplied to a plant growth vessel, filters are preferably provided in the inlet and outlet of the air supply to the vessel to maintain sterility within the vessel. The air supply may be passed through a filter, for example, an activated charcoal filter, to remove gaseous and/or volatile contaminants in the air supply. The filters used, particularly exhaust filters, are preferably hydrophobic as there is inevitable evaporation from the apparatus with the potential for condensation in the exhaust air stream. For long-term operation, it may be desirable to incorporate a condenser in the exhaust air stream to avoid build-up of condensation and potential microbial growth that may occur in filters, decreasing flow rates and possibly causing infection of the culture (filter "grow-through" phenomenon).

The plant growth vessel may be of any size and shape suitable for submerged liquid culture. Suitable vessels are well known for "air-lift" systems and for systems where the liquid medium is shaken or stirred. The vessel may be, for example, a flask, bottle, column or tank. The vessel may be of glass, metal or even a synthetic polymer, for example, polypropylene or polycarbonate. If the plant material is to be illuminated for photosynthesis, the vessel should allow the passage of light of the appropriate wavelength.

The liquid medium is introduced into the vessel, is preferably brought to the temperature at which cultivation will be carried out, the inoculum of shoots, rooted shoots or seedlings is added to the medium, and the apparatus is generally sealed. The plant growth vessel and its contents are maintained at an appropriate temperature, for example, from 20 to 30°C, with or without illumination.

In the case when air or another oxygen-containing gas is passed through the liquid medium in which the plant material is free to move, the supply is preferably adjusted to the maximum flow rate that gives a steady stream of bubbles such that the shoots, rooted shoots or seedlings are submerged and preferably tumble, and especially tumble freely, in the medium. The tumbling may be substantially continuous. In the case of systems in which the liquid medium is shaken or stirred, the shaking or stirring should be sufficient to oxygenate the medium while ensuring that the plant material is submerged.

An appropriate medium is chosen for the woody plant to be propagated, for example, KM medium (without gelling agent) may be used for the propagation of Eucalyptus grandis and hybrids thereof. Similarly, for other plants the gelling agent may be omitted from a solid or semi-solid medium previously used for the propagation of that plant.

In some cases it may be possible to use levels of phytohormones lower than are conventionally used in solid media, for example, in the propagation of Eucalyptus grandis according to the present invention, good results are obtained with 25% (or even less) of the conventional amount of BAP (6-benzylaminopurine).

When shoots of a woody plant comprising an apical meristem, for example, Eucalyptus grandis, are propagated according to the air-lift system of the present invention with the shoots tumbling freely in the liquid medium, the effects of correlative inhibition on axillary shoot meristems appear to be almost completely abolished. All the branch systems originating from existing nodes present at time of inoculation contain tertiary branches. Nodes of decreasing age resulting from extension growth from the apex of the inoculated shoot possess branch

systems of decreasing complexity. The shoot tips of the original stems, the primary branches and the secondary branches from both media all yield shoot tips that are well elongated, possess thick and robust stems and two or more well spaced nodes. There is remarkable degree of uniformity between the harvested shoots, regardless of the number of nodes that the source branches possessed at harvesting. Almost all of the tertiary branches consist of a shoot tip (two leaves and meristem) and no nodes.

When shoots are propagated in a submerged liquid culture system of the present invention where their movement is restricted, for example, by being held in a perforated container, for example, a cage or bag, within the plant growth vessel, the resulting shoots display substantially the same characteristics as described above for shoots that are able to move freely. They display a high degree of uniformity and are of good quality. Furthermore, the multiplication rates are generally as high as those for the freely moving shoots. It appears that a high shoot inoculation density may lead to a high multiplication rate.

Contrary to expectation, symptoms of hyperhydricity (vitrification) of leaves, if they occur, are mild and are only apparent on well expanded, older leaves. Younger material (i.e. at the shoot tips and apical nodes) does not show any significant signs of hyperhydricity. It has been found that, should any signs of hyperhydricity be observed during use of the liquid culture technique of the present invention, such signs are generally not significant, are readily reversed during subsequent culture on solid medium, and hence have little or no effect on the subsequent propagation or rooting of the shoots.

Shoots harvested from the plant growth vessels root well and, for example in the case of E. grandis, rooting

efficiency may be better than that resulting from methods of propagation that utilise solid media. In the case of Acacia mangium, the resulting shoots can be rooted in compost and transferred directly to the greenhouse without the need for rooting on solid media. This is a most surprising advantage, which is very important commercially. The quality of the Rhododendron and eucalyptus shoots and their good rooting efficiency indicates that they, too, may be rooted directly in compost and transferred to the greenhouse without an intermediate rooting stage in semi-solid media. Indeed, the good quality of the material obtained according to the process of the present invention suggests that direct rooting in compost and transfer to the green house may be possible with other genera in addition to Acacia and Eucalyptus.

In summary, the process of the present invention yields an abundance of green, healthy, highly uniform shoots capable of rooting or further propagation. In many cases the shoots are sufficiently elongated that they can be rooted directly without further specific elongation steps. In some cases the shoots can even be rooted directly in compost and transferred to the greenhouse without an intermediate stage of rooting on semi-solid media.

Some plants, for example, olive, become chlorotic (yellow) when propagated on gelled media. In contrast, when propagated according to the liquid culture system of the present invention, olive shoots are green and healthy. A further advantage is that the olive shoots are well elongated. In contrast, when propagated on gelled media, olive shoots require an extensive elongation period, usually of about a month, before rooting.

The extension rates of new shoots propagated using

the liquid culture system of the invention appears to be unusually uniform. For commercial micropropagation, uniformity of product is extremely important. Any developmental heterogeneity of shoots set for rooting is often amplified during subsequent development of the plants. Such heterogeneity is usual in woody plants, resulting in the need for grading of products and possible interruptions of supply.

This problem is illustrated in the commercial production of rhododendrons. In all the gelled systems used for rhododendron production, large amounts of callus may be formed on the base of the multiplying shoots. Adventitious shoots are often produced from this callus via organogenesis. These adventitious shoots are frequently not true to type (i.e. are abnormal) due to the phenomenon of somaclonal variation. (This phenomenon is often associated with changes in chromosome number or chromosomal rearrangements.) Currently, commercial rhododendron producers have to sacrifice multiplication rates in order to avoid callus and adventitious shoot formation to ensure that the plants they are propagating are true to type. The liquid system of the present invention avoids this problem as the uniformity of the resulting shoots is excellent and far superior to that obtained using gelled propagation systems. In particular, no adventitious shoots are produced, all new growth resulting from development of axillary buds.

Furthermore, as indicated above, Acacia shoots can be rooted in compost and transferred directly to the greenhouse without the need for rooting on solid media. This surprising advantage is very important commercially.

There are also major cost advantages compared to propagation systems that use solid media, including savings in manual labour (or the high capital costs of automating processes using solid media), disposables and

time. For instance, to produce 50 E. grandis shoots from a single starting shoot using typical solid media protocol requires three subculture steps (and associated media preparation and use of disposable culture dishes) and takes three months. This compares to a single culture step and one month using the system of the present invention.

A further advantage has been found when propagating Acacia and Eucalyptus according to the present invention. Acacia shoots form in clumps that can be dissected out to give individual rooting shoots. The remains of the clumps, consisting of branched stems but without shoot tips, may be reintroduced into liquid culture medium according to the process of the present invention, whereupon further shoots develop. This process may be repeated several times at least, without deterimental effect on the quality or quantity of the product.

With Eucalyptus, shoots may be harvested from a liquid system according to the invention, dissected from shoot masses and immediately reinoculated into plant growth vessels containing fresh media. The process may be repeated for several cycles at least, without any significant effect on the shoot multi-plication rates and without any deterioration in the quality of the shoots produced in successive cycles. It is considered that recycling of the propagation material in the process of the present invention will have general applicability, and may be used for other genera in addition to Acacia and Eucalyptus.

Recycling the propagation material results in considerable savings and a uniform product, so is particularly attractive commercially.

Thus, the system of the present invention is highly attractive commercially. The yields obtained are generally higher than those with gelled systems, and are

often much higher. The high quality of the product, both in the general health of the product and its remarkable uniformity, is a particular commercial attraction. For some species there are further specific advantages, as described above.

A further aspect of the present invention relates to the selection of shoots, rooted shoots or seedlings that have been modified genetically by the stable incorporation of one or more DNA sequences of interest, especially by the process of transformation, and that have a selectable characteristic, property or attribute, generally a selectable marker.

Methods that enable the introduction and stable incorporation of a DNA sequence of interest into plant material in order to achieve the desired genetic modification are well known and include Agrobacterium-mediated transfer and methods that introduce the DNA directly into cells, for example, electroporation of protoplasts, bombardment of embryos with DNA-coated particles and polyethyleneglycol-mediated gene delivery.

Agrobacterium-mediated genetic transformation methods are generally preferred to direct transformation with DNA for several reasons. The method is relatively fast and simple, relatively efficient and not expensive in terms of labour, materials or equipment. In addition, the majority of transformed plants produced using Agrobacterium-mediated transformation contain one or a low number of intact DNA inserts, and the inserted DNA is often stably expressed. Direct DNA transformation methods, such as those described above, frequently result in multiple copies of the DNA insert in the plant cells, and the inserts are often not intact due to rearrangements. Multiple copies of inserted DNA have been associated with instability of expression of the introduced (heterologous) genes. Hence, the advantages

of Agrobacterium-mediated over methods which employ direct DNA transformation are such that the latter are usually used only if the plant species is intransigent with respect to Agrobacterium-mediated transformation, for example, because those plant cells that are capable of regeneration into whole plants are recalcitrant to Agrobacterium-mediated transformation.

Selectable markers are well known and include, for example, genes that confer resistance to a selective agent, for example, an antibiotic or herbicide, or to another selective agent. Selection is generally carried out by growing the material that has been subjected to transformation on a medium that contains the selective agent, for example, the antibiotic, herbicide or other selective agent.

Selection is carried out on solid media i.e. solid or semi-solid gelled media. The process is generally slow and laborious. In the case of some plant species and/or when using some selectable marker genes and the appropriate selective agent, the process may be extremely slow, labour intensive and expensive.

The selection process involves culturing explants that have previously been transformed using either Agrobacterium-mediated or direct transformation techniques and in which a proportion of the cells contain introduced genes, on a medium containing an appropriate selective agent. The conditions and concen-tration of the selective agent are generally chosen such that there is inhibition of growth and/or development of untrans-formed cells and tissues. Cells containing a DNA insert containing the appropriate selectable marker gene continue to grow and/or develop and can therefore be identified. Alter-natively, transformed and untransformed cells may be differentiated on the basis of different phenotype when in contact with the selective agent, for

example, on the basis of the amount of pigment production or altered growth rate.

Optimally, the conditions and concentration of selective agent are chosen such that the transformed and untransformed cells and tissues may be differentiated after a short period of culture. In practice, however, the presence of the selective agent in sufficient concentrations to differentiate between transformed and untransformed cells and tissues may also slow or delay the growth and/or development of the transformed cells and tissues when compared to growth and development under non-selective conditions. That may be due to one or more factors, including the selectable marker gene giving rise to sub-optimal levels of resistance or tolerance to the selective agent, or to indirect effects.

Indirect effects may include the selective agent affecting the growth and development of untransformed cells or tissues surrounding transformed cells or tissues. The normal growth or development of untransformed cells or tissues may be required for these surrounding cells tissues to make a contribution to the normal growth and development of the transformed tissues (nurse effects). Indirect effects may also be due to release of toxic or inhibitory substances from the untransformed cells or tissues, which then have a deleterious effect on the transformed cells and tissues.

For example, when selecting on 10-25 mg l^{-1} G418 (geneticin), it can take up to six months to produce shoots of transgenic Eucalyptus grandis (or hybrids thereof) from leaf explants derived from field-grown clones on solid media after trans-formation with a disarmed Agrobacterium strain containing a plant-expressed NPTII gene. In contrast, the time taken for the production of untransformed shoots on similar culture medium lacking G418 is six weeks. Hence although the

presence of the selective agent enables transgenic shoots to be produced and inhibits the production of non-transgenic shoots, the presence of the selective agent may significantly slow the process by which the transgenic shoots are produced.

In addition to the delay in the production of transgenic shoots, the yield of transgenic shoots is also known to be reduced. For example, in a method for the production of shoots of transgenic Eucalyptus grandis (or hybrids thereof) from leaf explants on solid media after transformation with a disarmed Agrobacterium strain containing a plant-expressed NPTII gene the yield of transgenic plants is significantly lower when selecting on 10-25 mg l^{-1} G418 (geneticin) than when transgenic plants are produced on a similar medium lacking selective agent.

Furthermore, the quality of plants produced in tissue culture after extended periods of culture may also be poor, particularly if their production requires extended periods of culture of callus tissues. There are many examples in the literature where such extended periods of growth can result in the production of abnormal plants due to the phenomenon of somaclonal variation (abnormalities which are heritable and may be the result of changes in the number or structure of heritable elements within the cells of the plant being produced). Any reduction in the period of time spent in tissue culture should therefore reduce the risk of producing transgenic plants that are abnormal.

By way of example, methods as described above for selecting transformed material and producing rooted shoots of each genetically modified line (i.e. all plants derived from a single transformed cell) of a field-derived (clonal) Eucalyptus grandis (or a hybrid thereof) suitable for weaning and further growth in the greenhouse

or field take about 11 months to produce 100 shoots.

As an alternative to using a selectable marker gene and a selective agent, transgenic plants from non-selected populations may be detected using methods that are based on the detection of the introduced DNA sequence (transgene). Such methods include detection of the transgene itself using PCR, and detection of a product of the transgene. However, such methods, for example PCR, are themselves extremely time-consuming, labour intensive, and expensive. Those disadvantages are compounded because the events of transformation and regeneration that give rise to transgenic plants are relatively rare, so large populations of shoots or plants must be screened in order to detect those plants that have been transformed.

The present invention provides a process for selecting genetically modified shoots, rooted shoots or seedlings that comprise one or more stably incorporated DNA sequences of interest and that have a selectable characteristic, property or attribute, wherein the shoots, rooted shoots or seedlings are cultivated submerged in an oxygenated, for example, aerated liquid culture medium that comprises means for selecting the genetically manipulated shoots, rooted shoots or seedlings. The medium is generally agitated or otherwise moving.

The shoots, rooted shoots or seedlings may be allowed to move in the liquid medium, for example, they may be allowed to move freely, for example, to tumble, for example, tumble freely, in the liquid medium. Alternatively, their movement may be restricted or otherwise impeded.

The total immersion of the shoots, rooted shoots or seedlings in a solution of the selective agent results in more effective and more extensive penetration of the

selective agent into the plant tissues and hence enables effective selection of the genetically modified shoots, rooted shoots or seedlings.

Figure 2 of the accompanying drawings illustrates the typical steps and time-scales required to produce genetically modified Eucalyptus grandis clones (or hybrids thereof) using G-418 selection on solid (gelled) media and using the process of the present invention. Using solid media it takes about 47 weeks to produce about 100 shoots. Using the method of the present invention, more than 100 shoots are produced in 21 weeks.

Figure 3 of the accompanying drawings is a map of a plasmid identified herein as pSCV1, which is used in the production of plasmid pSCV1.6. Figure 4 is a map showing the T-DNA of a plasmid identified herein as pSCV1.6. Plasmid pSCV1.6 is used to introduce a DNA sequence of interest into Eucalyptus using Agrobacterium-mediated transfer.

The liquid medium may be both agitated and oxygenated by passing oxygen or, more usually, air, through the medium. Air circulation techniques, sometimes called "airlift" techniques, are particularly useful for providing simultaneous oxygenation and agitation in the process of the present invention. Liquid medium in an appropriate vessel, for example, a fermentation vessel, for example, a flask, bottle, tank or column may be circulated and oxygenated by the introduction of air through, for example, a gas diffuser. Such vessels are often called "air-lift fermenters". The volume of air and the rate of introduction can easily be adjusted to give the desired degree of agitation to the liquid medium and hence to the plant material when it is allowed to move freely.

When the plant material is allowed to move freely, agitation of the medium by passing air or another oxygen-

containing gas through the medium may be preferable to agitation by mechanical means, for example, stirring, because the shearing forces on the plant material may be lower. However, the shearing forces on the plant material in shaken or stirred systems of the present invention may by reduced by restricting the movement of the plant material, for example, as described in more detail below. In a system where air or another gas is passed through the medium it may also be desirable to restrict the movement of the plant material, so the liquid medium is agitated but the plant material is restrained.

If the passage of the gas is not sufficient to achieve the desired agitation, additional agitation means may be provided, for example, the plant growth vessel may be also be shaken or the contents may be stirred. The vessel may be illuminated to provide further oxygen by photosynthesis.

The selection process of the present invention has universal applicability, that is to say, it may be applied to selection of genetically manipulated shoots, rooted shoots and seedlings of any plants. The plants may be, for example, annual, biennial or perennial plants; they may be monocotyledonous or dicotyledonous plants; they may be herbaceous or woody plants, for example, woody plants as described above.

Woody plants that appear to perform particularly well in the liquid culture system of the present invention are sclerophyllous species. The definition of sclerophyll is "thick, leathery leaf". This includes true leaves, as in the case of eucalypts and phyllodes as in the case of some Acacias. Species considered as sclerophyllous are generally evergreen and the sclerophyllous habit is generally associated with poor nutrient availability and often with drought tolerance. Examples of sclerophyllous

genera are Rhododendron, Azalea and Kalmia (Ericaceae); Olea (Oleaceae); many Australian Acacias (Fabaceae); and eucalypts (Myrtaceae).

However, it is not only sclerophyllous species that perform well in the liquid culture system of the present invention. Malus (apple), Pyrus, Prunus and Rosa (Rosaceae), Forsythia and Syringa (Oleaceae) are further examples of woody plant genera that may be propagated using the liquid culture system of the present invention.

As indicated above, eucalyptus is an example of a sclerophyllous woody plant that may be selected according to the present invention. The sub-genus Eucalyptus symphyomyrtus contains many commercially useful species, for example, E. grandis, E. globulus, E. nitens, E. dunnii, E. saligna, E. camaldulensis, E. urophylla and hybrids thereof. Further commercially important Eucalyptus species include E. regnans, E. citriodora, E. fraxinoides and hybrids thereof.

According to the selection process of the present invention, genetically modified shoots, rooted shoots or seedlings that comprise one or more stably incorporated DNA sequences of interest and that have a selectable characteristic, property or attribute, are cultivated submerged in an oxygenated liquid medium that comprises means for selecting the genetically manipulated shoots, rooted shoots or seedlings.

The DNA sequence(s) of interest may be heterologous to the recipient plant, or may be homologous. They must be functional in the recipient plant. Many examples of DNA sequences of interest in the genetic modification of plants are known. For example, the DNA may function to impart to the recipient plant a phenotypic property, e.g. resistance to a herbicide such as glyphosate, to modify the quality or the chemical components of the plant, to modify rooting ability of vegetative propagules, for

example, cuttings, or to confer reproductive sterility.

Methods that enable the introduction and stable incorporation of a DNA sequence of interest into plant material in order to achieve the desired genetic modification are well known and include Agrobacterium-mediated transfer and methods that introduce the DNA directly into cells, for example, electroporation of protoplasts, bombardment of embryos with DNA-coated particles and polyethyleneglycol-mediated gene delivery. Agrobaterium-mediated transfer is generally the method of choice, for the reasons given above.

As discussed above, successful transformation of cells or tissue, for example, shoots, is generally determined using a suitable characteristic, property or attribute as a marker, especially a selectable marker gene. Selectable marker genes and corresponding selective agents for use with solid culture systems are well known and are described, for example, in the literature of this art. Any such selectable maker gene and corresponding selective agent may be used in the submerged liquid culture selection process according to the present invention.

For example, the NPTII gene may be used as a marker gene, with resistance to a phytotoxic selective agent conferred by that gene, for example, resistance to paromomycin, G-418 (also known as geneticin) neomycin or kanamycin used as the characteristic for selection of transformed cells or tissue. Any other DNA sequence that confers the same or similar resistance may be used as the selectable marker.

The total immersion of the shoots, rooted shoots or seedlings in a solution of the selective agent results in more effective and more extensive penetration of the selective agent into the plant tissues and hence enables effective selection of the genetically modified shoots,

rooted shoots or seedlings.

The selective agent, for example, a herbicide or antibiotic, for example, paromomycin, G-418 or neomycin, should be used in the liquid medium in a concentration and in a regime that enables effective selection of the transformed shoots and seedlings. Examples of suitable concentrations and regimes are given herein. Optimal conditions for any selected system may be determined readily.

Suitable solid media and conditions for plant culture are known. Optimal liquid media and culture conditions for any particular plant starting material may be determined by routine methods if they are not already known. For example, the formulation for a liquid medium for a particular plant may be based on the formulation of a solid medium known for that plant.

According to the present invention, the genetically modified shoots, rooted shoots or seedlings are cultivated under submerged conditions in an oxygenated liquid medium that comprises the selective agent, for example, the antibiotic, herbicide or other selective agent. The cultivation of the genetically modified shoots, rooted shoots or seedlings in the oxygenated liquid culture medium is preferably carried out as described above for micro-propagation of shoots, rooted shoots and seedlings. After selection according to the process of the present invention, the genetically manipulated plants may be further propagated, rooted or cultivated as desired.

It is a particular advantage that the selected material is already in the form of rooted or readily rootable shoots, or as seedlings, rather than as a primordial mass, as in conventional selection methods.

There is the further advantage that the shoots, rooted shoots or seedlings resulting from the selection

in the oxygenated liquid medium are of high quality, showing particularly uniform growth, as described above for the products of micropropagation according to the invention. This is particularly useful for the further cultivation of the selected plant material.

The most dramatic advantages over conventional selection methods are the speed at which genetically modified plant material can be selected, and the reduction in costs, including both labour and materials.

As stated above, the selection process of the present invention has universal applicability, that is to say, it may be applied to selection of genetically manipulated shoots, rooted shoots and seedlings of any plants. The plants may be, for example, annual, biennial or perennial plants; they may be mono-cotyledonous or dicotyledonous plants; they may be herbaceous or woody plants, for example, the sclerophyllous and other woody plants specifically described above. It is generally advantageous to use, as starting material for genetic manipulation, plant cells or tissues that are genetically uniform, for example, cells or tissue derived from homozygous seed or clonal material that is vegetatively derived, directly or indirectly, from vegetative tissues of plants that have been selected, or are selectable, for favourable characteristics.

However, in some cases, for example, woody plants, especially trees, for example, Eucalyptus, a desired characteristic can only be assessed in a mature plant, but clonal material obtained from mature plants is often difficult to modify genetically and/or recalcitrant to shoot induction. This is particularly the case in woody plants, especially trees, for example, Eucalyptus.

A particular embodiment of the present invention enables cells and tissue derived via vegetative propagation i.e. clonal material, especially clonal

material from plants exhibiting superior phenotypic properties, to be modified genetically, selected and regenerated into viable plants, rapidly and in high yields.

In that embodiment, cells or tissue of a plant are subjected to Agrobacterium-mediated transfer of one or more DNA sequence(s) of interest, shoot formation is induced in the resulting transformed cells or tissue, which have a selectable characteristic, property or attribute, in the presence of an agent capable of inducing shoot formation in that plant, and the resulting shoots are selected in an oxygenated liquid culture medium that comprises means for selecting the genetically modified shoots, the shoots being submerged in the liquid medium. The selected, transformed shoots may then be regenerated into viable plants.

The shoot inducing agent should be capable of inducing, preferably at high frequency, the formation of buds that are capable of further development. Such agents are generally cytokinins. The suitability of a particular agent for any particular plant starting material and appropriate concentrations of the selected agent and regimes for its use may be determined by routine methods. The agent is incorporated in the culture medium used for shoot induction and preferably also in the liquid medium used for the selection of transformed shoots. Two or more agents may be used.

The cytokinin BAP is a suitable shoot induction agent for many plants, for example, apples and poplar. A further example of a shoot inducing agent for use according to the present invention is the substituted phenylurea N-(2-chloro-4-pyridyl)-N'-phenyl-urea, often known as 4-PU or CPPU. CPPU has been found to induce bud formation in Eucalyptus and other plants at high frequency and, unlike some other phytohormones and plant

growth factors, a further effect is that the buds produced are capable of further development into shoots. Other substituted phenylureas may be used instead of or in addition to CPPU for the selected plant material, for example, Eucalyptus, provided they are capable of inducing, preferably at high frequency, the formation of buds that are capable of further development.

This embodiment of the present invention has universal applicability, that is to say, it may be applied to any plants. As explained above, it is particularly useful for the genetic manipulation and subsequent selection of clonal material obtained from plants that are intransigent to transformation and/or shoot induction, for example, woody plants, for example, trees. Examples are sclerophyllous and other woody species as described above, for example, Eucalyptus.

The cell or tissue material, especially cells and tissue derived via vegetative propagation i.e. clonal material, especially clonal material from plants exhibiting superior phenotypic properties, may be obtained directly from a plant grown in the field or a greenhouse. It may be used in non-sterile form, i.e. without the use of an intervening micropropagation step, for the introduction of heterologous (or homologous) gene(s). Alternatively, the cells or tissue may be derived indirectly from selected plants that is to say, the cells or tissue taken from the selected plant is subjected to micropropagation before genetic manipulation.

In the case of clonal material, the starting material may be obtained from any plant of interest. The plant may be a mature tree, for example, Eucalyptus. In the case of Eucalyptus, it may be obtained, for example, from a member of the sub-genus Eucalyptus symphyomyrtus, for example, from E. grandis, E. dunnii, E. saligna,

E. camaldulensis, E. urophylla, E. regnans, E. citriodora or E. fraxinoides, or from a variety, cultivar or hybrid thereof.

The cells or tissue used as starting material for genetic modification according to the present invention may be derived from seedlings, especially young seedlings. The process of the present invention is particularly useful for the genetic modification of cells and tissue obtained from seedlings within the sub-genus Eucalyptus symphyomyrtis, for example, E. globulus, E. nitens and E. dunnii seedlings.

Any appropriate Agrobacterium vector may be used to mediate genetic modification of the plant material, for example, Agrobacterium tumefaciens or Agrobacterium rhizogenes. The Agrobacterium tumefaciens strain used to transform E. grandis clones, E. grandis/E. camaldulensis hybrid clones and E. saligna/E. tereticornis hybrid clones as described in the Examples is the disarmed strain EHA101A containing the binary Ti plasmid pSCV1.6. Figures 3 and 4 of the accompanying drawings are maps relating to plasmid pSCV1.6. Strain EHA101A may be used for the transformation of other Eucalyptus and also of any other plants. Examples of binary Agrobacterium-Ti plasmid vector systems have been fully described elsewhere, e.g. in EP-A-0120516.

As set out above, the shoot inducing agent or mixture of agents should induce, preferably at high frequency, the formation of buds that are capable of development into shoots. For Eucalyptus, which is particularly recalcitrant to undergo shoot induction, CPPU or an equivalent substituted phenylurea is particularly preferred.

The culture medium used for induction of shoot formation may contain glutamate and/or ascorbic acid, in order to promote regeneration of shoots at high

efficiency. The starting pH may be 5.0-5.6. The induction of shoot formation is generally carried out by culture on a solid medium or using another static culture medium. Examples of media suitable for use in the process of the present invention for inducing shoot formation, for selection of transformed cells and tissue and for multiplication and inducing root formation of genetically modified Eucalyptus are given herein. Suitable media for many other plants are known or may be determined by routine methods.

The selection and, where desired, cultivation of transformed shoots may be carried out as described above, preferably under using the apparatus and conditions as described above for micropropagation of shoots, rooted shoots and seedlings.

Figure 2 of the accompanying drawings illustrates, by way of a non-limiting example, steps involved in a typical process for the production of rooted genetically modified plants using selection on solid (gelled) media, and carrying out selection in a liquid medium using a submerged "air-lift" culture according to the present invention, in which the plant material is allowed to move freely. The time scale of the two processes and the media used are described below by way of example for the production of genetically modified Eucalyptus grandis hybrid plants. It will be appreciated that using the protocol illustrated in Figure 2, the time scale and media of choice will depend on the nature of the plant material to be modified genetically. Furthermore, the steps illustrated may be carried out in a different order, steps may be omitted and/or other steps added. Any of the other liquid systems of the present invention may be substituted for the "air-lift" system, with similar results.

In Step 1, which is common to both methods, explants

for example of Eucalyptus grandis hybrids are transformed with a DNA sequence of interest using Agrobacterium-mediated trans-formation so that the transformed material will also contain a selectable marker gene, for example, the NTPII gene.

In Step 2, which is also common to both methods, the resulting transformed explant and Agrobacterium strain are co-cultivated for 2 days on a solid (gelled) medium, for example, clonal co-cultivation medium containing a shoot inducing agent. For Eucalyptus it is particularly advantageous to use CPPU to induce shoot formation.

In Step 3, the resulting explants previously challenged with the Agrobacterium strain are regenerated to produce shoots on solid (gelled) medium containing a shoot inducing agent. For Eucalyptus, it is again advantageous to include CPPU in the solid medium to induce shoot formation. The solid medium also contains the appropriate selective agent. The solid medium preferably contains an antibiotic to prevent the growth of the Agrobacterium, for example, augmentin. For selecting transformants containing the NTPII gene the selective agent is, for example, paromomycin, G418, kanamycin or neomycin. For Eucalyptus the solid medium is, for example, clonal cocultivation medium with CPPU and augmentin, and also containing G418, for example 25 mg/l G418. For Eucalyptus, subculture is carried out every three weeks until shoots appear. This step takes about 21 weeks using a medium containing CPPU, augmentin and G418.

Step 3 according to the present invention involves shoot induction without selection. The explants resulting from Step 2, which explants have previously been challenged with the Agrobacterium strain are cultured on a solid (gelled) medium containing containing a shoot inducing agent. For Eucalyptus it is

particularly advantageous to use CPPU to induce shoot formation. The medium preferably also containing an antibiotic, for example, augmentin to prevent the growth of the Agrobacterium. As for the conventional method, subculture is carried out every three weeks until shoots appear. In this case, however, shoots appear after 4-5 weeks and are allowed to continue development up to a total of about 9 weeks, rather than 21 weeks in the conventional process. This is a saving of 12 weeks and 4 subculture steps, with corresponding savings in labour and materials.

Step 4 involves elongation of the selected shoots on solid (gelled) medium, for example, clonal shoot elongation medium, which takes about 6 weeks. In the process of the invention, in Step 4 selection of transformants and shoot elongation is carried out simultaneously in submerged liquid culture using an "air-lift" system in which the shoots are allowed to move freely, as described above. The liquid medium contains an appropriate selective agent, for example, paromomycin for the NPTII gene and, preferably also an antibiotic, for example, augmentin. In this case selection and shoot elongation takes less than 16 days. The non-transformed material rapidly becomes brown, for example, the first signs show within 4 to 8 days. Shoots that are totally transformed, in contrast, are healthy and green and the shoots rapidly elongate. Shoots that are composed of both transformed and non-transformed tissues (chimaeric shoots) are easily differentiated from shoots that are composed entirely from transformed cells either by the presence of brown sectors or by partial browning over the entire surface of the shoot. The totally transformed shoots are highly uniform and of very good quality. After 10 days, high quality transformants are available for micropropagation. The selective agent is able to

penetrate the plant tissues more effectively and more extensively in the submerged liquid culture according to the process of the present invention than is possible when the selective agent is present in a solid medium.

Micropropagation of the shooted transformants is carried out in Step 5. Using a solid (gelled) medium, for example, solid KM medium, it takes about 16 weeks to obtain about 100 Eucalyptus grandis shoots of each genetically modified line of shoots i.e. derived from a single transformation event. Using a submerged "air-lift" liquid culture according to the present invention in which the shoots are allowed to move freely, for example, using liquid KM medium, more than 100 shoots are obtained in about 6 weeks. As explained above, this results in savings in labour and materials as well as in time.

The final step, Step 6, is common to both methods. It involves rooting the shoots on solid (gelled) medium, for example, KM medium with IBA. In both cases it takes about 4 weeks.

The present invention reduces the time needed to produce a population of 100 rooted transformed Eucalyptus plants by six months, and saves in the order of eight manual handling (sub-culture) steps, resulting in large savings in the costs of labour, media and disposable items such as plant growth vessels. The process of the present invention also reduces costs significantly as the amount of facilities needed to handle the cultures under aseptic conditions and incubate the cultures under controlled environmental conditions is significantly reduced.

Not only are more shoots obtained in less time at lower cost than when using solid media for selection, the resulting shoots are of very much higher quality. In particular, as described above for micropropagation

according to the present invention, the shoots are well elongated, posses thick and robust stems and two or more well spaced nodes. Furthermore, the shoots are remarkably uniform, which is especially important for commercial purposes.

If desired, a selection process of the present invention, that is to say, selection in a liquid medium, may be incorporated in a protocol in which a selection step on solid (gelled) medium is carried out before and/or after selection in the liquid medium. In such a case the selection on the solid medium may be less stringent than would be required if the solid selection step were the only selection step. A reduction in stringency reduces adverse effects on growth and development that may be caused by stringent selection conditions.

The present invention accordingly provides a process for selecting genetically modified shoots, rooted shoots or seedlings that comprise one or more stably incorporated DNA sequences of interest and that have a selectable characteristic, property or attribute, wherein the shoots, rooted shoots or seedlings are cultivated submerged in an oxygenated liquid medium that comprises means for selecting the genetically manipulated shoots, rooted shoots or seedlings before and/or after the shoots, rooted shoots or seedlings are cultivated on a solid, that is to say gelled, medium that comprises means for selecting the genetically manipulated shoots, rooted shoots or seedlings.

Genetically modified shoots, rooted shoots or seedlings selected according to a process of the present invention may be further micropropagated according to the micropropagation process of the present invention.

The present invention also provides genetically modified plants obtained from the shoots, rooted shoots

or seedlings selected and, optionally, further micropropagated according to a process of the present invention. Such plants may themselves be micro-propagated according to the micropropagation process of the present invention. As indicated above, this is particularly useful for the clonal propagation of mature trees, for example, eucalypts.

Microbial contamination is a potential problem in tissue culture systems in general. It may be a particular problem when the starting material has been obtained from plants grown under non-sterile conditions, for example, in a glasshouse or outdoors, even though surface sterilisation or disinfection is carried out according to conventional methods. Even when the starting material has been produced under sterile conditions there may be microbial contamination from other sources. It is also arises in connection with genetically manipulated plant material obtained using Agrobacterium-mediated transfer. Such material may present particular problems for subsequent propagation, particularly micropropagation, because of the inevitable contamination with the Agrobacteria themselves. Plants contaminated with Agrobacterium may also be unsuitable for commercialisation because of regulatory considerations.

The present invention provides a process for reducing microbial contamination of shoots, rooted shoots or seedlings, which comprises cultivating the shoots, rooted shoots or seedlings in an oxygenated liquid medium that comprises an antibiotic, the shoots, rooted shoots or seedlings being submerged in the liquid medium.

The present invention also provides the use of cultivation of shoots, rooted shoots or seedlings in an oxygenated liquid medium that comprises an antibiotic, the shoots, rooted shoots or seedlings being submerged in

the liquid medium, for the reduction of microbial contamination of the shoots, rooted shoots or seedlings.

The liquid culture medium employed comprises an antibiotic, for example, augmentin. The fact that the shoot, rooted shoot or seedling masses are totally immersed in the liquid medium results in more efficient penetration of antibiotic into the tissues of the shoot masses than occurs on solid media, and hence microbial contamination may be reduced or even removed in a simple and effective manner.

The cultivation of the shoots, rooted shoots or seedlings in the oxygenated liquid culture medium is preferably carried out as described above for micropropagation of shoots, rooted shoots and seedlings.

The shoots or rooted shoots may have been obtained from plants grown under non-sterile conditions, for example, in a glasshouse or outdoors, and may have been subjected to surface sterilisation or disinfection as carried out according to conventional methods. Similarly, seedlings may have been grown under non-sterile conditions, for example, in a glasshouse or outdoors, and may have been subjected to surface sterilisation or disinfection. The shoots, rooted or seedlings may have been obtained from genetically manipulated plant material obtained using Agrobacterium-mediated transfer.

The process of the present invention for reducing microbial contamination has universal applicability, that is to say, it may be applied to selection of genetically manipulated shoots, rooted shoots and seedlings of any plants. The plants may be, for example, annual, biennial or perennial plants; they may be gymnosperms, monocotyledonous or dicotyledonous plants; they may be herbaceous or woody plants, for example, the sclerophyllous and other woody plants specifically described above. The plants may of use in agriculture, horti-

culture, forestry or as plantation crops. The plants may be ornamental or produce useful crops or products.

The shoots, rooted shoots or seedlings obtained by any process of the invention described herein may be grown into plants, for example, mature plants, under appropriate conditions, for example, in a greenhouse or outdoors. It is an advantage that, in many cases, shoots obtained according to a process of the invention may be rooted directly into compost and grown up in a greenhouse, rather than requiring an intermediate stage of rooting on semi-solid media.

As set out above, depending on the embodiment of the invention, the plants may be, for example, annual, biennial or perennial plants; they may be gymnosperms, monocotyledonous or dicotyledonous plants; they may be herbaceous or woody plants, for example, the sclerophyllous and other woody plants specifically described above. The plants may of use in agriculture, horticulture, forestry or as plantation crops. The plants may be ornamental or produce useful crops or products. Examples of plants and their uses are given above.

Plants obtained from shoots, rooted shoots or seedlings obtained by any process of the present invention are themselves part of the present invention, as are products obtained from such plants. Such plants may be micropropagated according to a micropropagation process of the present invention. For the reasons given above, this is particularly useful for the propagation of mature plants, for example, mature eucalypts.

The following non-limiting Examples illustrate the invention.

EXAMPLES 1 TO 10

PROPAGATION OF E. GRANDIS AND E. GRANDIS HYBRIDS

Unless specified otherwise the media, plant

materials, temperature, airflow and lighting conditions described below were used in the following Examples 1 to 10:

Media

Solid KM media for micropropagation of E. grandis & E. grandis hybrids

Phytagel	3 g/l
Sucrose	10 g/l
10 X Macronutrient solution[1]	100 ml/l
$MgSO_4.7H_2O$	0.925 g/l
NH_4NO_3	0.825 g/l
Murashige and Skoog (1962)[2] basal salt micronutrient stock solution (Sigma M0529)	50 ml/l
1000 X Murashige and Skoog (1962) vitamin solution (Sigma M03900)	0.5 ml/l
BAP (6-benzylaminopurine)	0.04 mg/l

Adjust pH 5.6. with KOH and autoclave at 121 °C for 20 minutes.

[1]10 X macronutrient solution contains 2.2 g/l $CaCl_2.2H_2O$, 0.85 g/l KH_2PO_4 and 1.9 g/l KNO_3

[2]Murashige T; Skoog F: (1962) A revised medium for rapid growth and assay with Tobacco tissue cultures. Physiol. Plant., 15 473-497.

Liquid KM media for micropropagation of E. grandis & E. grandis hybrids

Ingredients: as for the solid KM medium, except that

(i) the Phytagel is omitted and

(ii) 0.01 mg/l BAP are used instead of 0.04 mg/l BAP that is used in the solid medium.

Half-strength KM medium for rooting of shoots of E. grandis and E. grandis hybrids

This medium contains half the macronutrient, micronutrient, $MgSO_4.7H_2O$ and NH_4NO_3 content of the basic solid KM medium. BAP is omitted and replaced with 0.2 mg/l IBA (indoyl-3-butyric acid). The pH is adjusted

to pH 5.6 and the medium is autoclaved at 121 °C for 20 minutes as described above for the full strength basic KM medium.

Where applicable, 1.0 g/l activated charcoal is added prior to sterilisation.

Temperature, airflow and lighting conditions

Liquid micropropagation, micropropagation and rooting on semi-solid media were conducted at 22 °C with a 16 hour photoperiod (50-70 µmol m^{-2} s^{-1}, supplied by fluorescent lamps). Unless specified otherwise, airflow rates were in the range of from 0.3-0.7 litre per minute per litre of liquid medium.

Plant material used for inoculation

All plant materials used for inoculation were previously micropropagated on semi-solid media. *E. urophylla* x *grandis* hybrid clones 18.50 and 18.52 were obtained from Centre National de Recherches Forestieres, B.P. 764, Pointe Noire, Republique Du Congo.

E. grandis clones 5046 and 5048 were obtained from Prof D.L. Rockwood, Dept. Forestry, Univ. Florida, Gainesville, Florida 32611-0303 USA.

E. grandis x E. camaldulensis hybrid clone 11/25 was supplied to Shell South Africa by the South African Forestry Research Institute, PO Box 727, Pretoria 0001, South Africa.

Micropropagated E. grandis seedling line T14 L10 was derived from seed of E. grandis supplied by the Institute of Commercial Forestry Research, University of Natal, P.O. Box 375, Pietermaritzberg 3200, Republic of South Africa (seed batch reference No. 38064).

Preparation of shoots used for inoculation

Micropropagated shoots of E. grandis or E. grandis hybrids having apical meristems were used as starting material. Each shoot possessed 2-4 nodes and a shoot tip. The shoots had previously been serially subcultured

on basic solid KM medium containing 0.04 mg/l BAP. The cultures from which the shoot tips were harvested had rooted spontaneously. The plant material from which the shoots were derived had previously been cultured for extensive periods on antibiotic (augmentin)-containing media and was known to be free of microbial contaminants.

Multiplication rates of shoots

Unless specified otherwise, the multiplication (propagation) rates given in the Examples are for shoots that are suitable for rooting either directly into compost or indirectly via semi-solid media.

EXAMPLE 1

Propagation of E. grandis shoots using an airlift system with free movement of shoots.

The media, temperature, airflow and illumination conditions used were those set out above.

Apparatus

The apparatus consisted a compressed air supply fed via a combined pressure regulator and gauge. A pressure release valve (NUPRO SS-6C-MM-10), activated when the pressure reaches approximately 10 p.s.i. (approximately 67,000 Pa) was fixed into the line downstream of the regulator. The airstream was passed through an activated charcoal gas filter (Whatman Carbon Cap 75) and humidified by passage through distilled water using a gas diffuser (Pyrex no. 2) and a 2l Erlenmayer flask. The deodorised and humidified air stream was used to supply an air-lift fermenter. The airstream was sterilised by passage through a gas filter (Whatman Hepa-Vent 0.3 µm pore size) before being fed into the air-lift fermenter. The air-lift fermenter comprised a 5l culture vessel (Quickfit FV) fitted with a lid (Quickfit MAF2/2) having five 19 mm ports. The airstream was passed through the central port using 6mm diameter stainless steel tubing and a lid adaptor (Quickfit). A gas diffusion tube

(Pyrex no. 2) was attached to the stainless steel inlet so that the diffuser was approximately 0.5 cm from the base of the assembled culture vessel. The exhaust consisted of a stainless steel tube, inserted into a port using a screwjoint adaptor (Quickfit) vented to the atmosphere via a gas filter (Whatman Hepa-Vent).

Silicone tubing (tissue culture grade, Merck) was used throughout the apparatus downstream of the activated charcoal filter. It is possible to replace the glass culture vessels by plastics (tissue culture grade) bottles, for example, tissue culture grade polypropylene or polycarbonate bottles, which are considerably cheaper.

Preparation of the apparatus

The air-lift fermenter vessel was filled with 4.5l of the liquid KM medium described above. Silicone grease was used to ensure a good seal of the flange and the lid secured using a springclip (Quickfit JC 100F). Two of the remaining ports were securely stoppered. The inlet, exhaust ports and two stoppered ports were wrapped in foil and sealed with tape as an added precaution against these becoming potential routes for contamination. The remaining port was loosely stoppered and loosely wrapped in foil and tape to allow access of steam during sterilisation and to enable subsequent inoculation with shoots. The fermenter and inlet/exhaust filters were autoclaved as assembled units using a 30 min preheat/-60 min full pressure (121 °C) cycle. The fermenter was allowed to cool to room temperature (overnight) before the inoculation port was secured and the fermenter removed from the autoclave. The fermenter was placed in a 22 °C waterbath, connected to the air supplies and equilibrated for 1 hour prior to inoculation (air-flow rate approximately 1.8 litres/minute).

Micropropagation of shoots

On the first day, the fermenter was inoculated with

ten micropropagated E. grandis shoots obtained from the seedling line T14 L10. The total weight of the inoculum was 493 mg. The fermenter was reconnected to the air supplies. The air-flow was adjusted to the maximum flow rate that gave a steady stream of bubbles, approximately 1.8 l/minute. The fermenter was shielded with safety screens, and operated for a period of 27 days prior to estimation of multiplication rates and increase of biomass.

Biomass increase was estimated by removing three shoot masses, blotting dry onto filter papers and weighing. Moisture content was estimated by drying to constant weight in a vacuum oven at 60 °C for 72 hours. The percentage moisture content of micropropagated shoots similar to those used for inoculation was estimated using the same method. Multiplication rates were estimated by dissection of three shoot masses from each fermenter into nodal and shoot tip explants. The nodal explants and some of the shoot tip explants were subcultured onto two solid basic micropropagation media (solid KM media containing 0.04 mg/l BAP and/or solid KM media containing both 0.04 mg/l BAP and 1% w/v activated charcoal). Rooting efficiencies of shoot-tip explants were estimated by transfer onto two rooting media (solid half-strength KM media containing 0.2 mg/l IBA and solid KM media containing both 0.2 mg/l IBA and 1% activated charcoal). After 5 days, some of the shoot tips that were initially transferred onto rooting medium containing activated charcoal were subcultured onto rooting medium lacking charcoal.

<u>Results</u>

After initial inoculation, the surface of the shoots did not readily become wetted. Air bubbles bound to the shoots which floated on the surface of the media and did not tumble to any great extent. After several days, the

surfaces of the shoots became wetted and the shoots began to tumble extensively.

Nine days after inoculation, the shoots were showing high rates of extension growth at the apices and the majority of the axillary buds that were present at the time of inoculation had commenced growth. A few of the larger leaves present at the time of inoculation were showing signs of hyperhydricity (vitrification), necrosis and undergoing abscission.

Sixteen days after inoculation, the medium was starting to become turbid. Some signs of hyperhydricity, necrosis and abscission of older leaves was becoming apparent. Side-shoots were continuing to elongate. The new axillary buds resulting from extension growth from the apices and primary branches were starting to commence growth. All of the new growth appeared healthy with no abnormal development being apparent. Six of the shoot masses were developing roots from the base of the shoot masses.

Twenty-four days after inoculation, turbidity of the medium had increased and a scum-like deposit had formed on the inside of the vessel above the level of the media. Signs of hyperhydricity, necrosis and abscission of older leaves became more pronounced and was occurring on the larger leaves of the primary branches. Samples of the media were taken and examined microscopically in order to determine the cause of the turbidity. The medium contained both intact and disrupted plant cells and also large amounts of cell debris, but there was no evidence of microbial contamination. Overall, the shoot masses were compact, green and showed few signs of hyperhydricity. A few of the new primary and secondary branches had broken off the main shoot masses.

The shoot masses were harvested from the fermenter 27 days after inoculation. Figure 1 shows the appearance

of typical shoot masses from the fermenter in comparison with the typical shoot used as inoculum and with a typical shoot mass cultivated on solid media: The shoot mass 1 in Figure 1 is typical micropropagated shoot used as inoculum. The shoot mass 2 is a typical shoot mass produced after five weeks cultivation on solid micropropagation medium (KM medium). The shoot masses 3a and 3b are two typical shoot masses harvested after 27 days submerged liquid culture in liquid KM medium.

Estimates of increase in wet weight, dry biomass and moisture content of shoots harvested from fermenters are shown in Table 1 below.

TABLE 1

Wet weight, dry biomass and moisture content

- Wet-weight of inoculum (10 shoots)	493 mg
- Estimated dry weight of inoculum (at 91.8% moisture content)	40.4 mg
- Wet weight of 3 typical shoot masses harvested from fermenters	15.466 g
- Estimated increase in wet weight (corrected for total yield of 10 shoot masses)	104.5-fold
- Dry weight of three typical shoot masses harvested from fermenters	1.671 g
- Estimated dry weight increase (corrected for total yield of 10 shoot masses)	137.9-fold

Estimates of total multiplication rates and shoot-tip

multiplications rates are shown in Table 2 below. In that Table, estimates of micropropagation rates are expressed as shoot-tip explants which were judged suitable for directly setting for rooting and as total explants which were suitable for further micropropagation on solid media.

TABLE 2

Multiplication rates

Number of shoot-tip explants suitable for rooting from each of three shoot masses	59, 46, 56
Average	54
Estimated number of explants suitable for further micro-propagation from each of three shoot masses	111, 102, 121
Average	111

In the resulting shoots, the effects of correlative inhibition on axillary shoot meristems appeared to be almost completely abolished. All the branch systems originating from existing nodes present at time of inoculation contained tertiary branches. Nodes of decreasing age resulting from extension growth from the apex of the inoculated shoot possessed branch systems of decreasing complexity.

Tertiary branches had started to form from axillary buds on the secondary branches. The main stems had grown extensively and the shoot masses possessed an average of 10 nodes (range 9-11 on the three shoots dissected). The stems of the original explants had expanded to

approximately 2 mm diameter at the base and the primary, secondary and tertiary branches had decreasingly smaller diameter stems at their branch points. The shoot tips of the original stems, the primary branches and the secondary branches from both media all yielded shoot tips that were well elongated, possessed thick and robust stems and two or more well spaced nodes. Within a single branching class of shoots, there was a remarkable degree of uniformity between the harvested shoots, regardless of the number of nodes that the source branches possessed. Almost all of the tertiary branches consisted of a shoot tip (two leaves and meristem) and no nodes. The greatest difference between shoots of a single branching class was seen in the degree of elongation of the tertiary shoots. Only well elongated tertiary shoots were harvested for subsequent transfer to solid media. Roots were only observed developing from the base of seven of the main inoculated shoots.

Where observed, signs of hyperhydricity of leaves was apparent on well expanded, older leaves only and was not as advanced as was expected from observations made whilst the fermenter was in operation. Younger material (i.e. at the shoot tips and apical nodes) did not show any significant signs of hyperhydricity. Dissection of the shoot masses indicated that the older stems were rather brittle, indicating hyperhydricity.

After harvest from the shoot masses produced in liquid KM medium and subsequent transfer to solid KM micropropagation media, shoot-tip explants and nodal explants appeared healthy. Four days after transfer, no differences could be detected between explants transferred to KM micropropagation media containing or without activated charcoal. The explants looked healthy in comparison to similarly treated shoot-tip and nodal explants previously cultured on solid KM micropropagation

medium lacking activated charcoal. 28 days after transfer, the growth of apical meristems (where present), commencement of growth of new axillary meristems and subsequent elongation growth of axillary shoots appeared to be similar in explants either harvested from shoot masses produced in liquid KM medium or in explants previously cultured on solid KM micropropagation medium lacking activated charcoal.

Rooting efficiencies of shoot-tip explants harvested either from shoot masses produced in liquid KM medium or harvested from shoots previously cultured on solid KM micropropagation medium lacking activated charcoal are shown in Table 3.

TABLE 3

Rooting efficiencies of shoot-tip explants 28 days after transfer to rooting media

Percentage rooting of shoot-tip explants

Rooting Treatment	Shoot-tip explants harvested from shoot masses produced in liquid KM medium	Shoot tip explants harvested from shoot cultures produced on solid KM medium lacking activated charcoal
Half-strength KM rooting medium	86	66
Half-strength KM rooting medium plus activated charcoal	52	41
Half-strength KM rooting medium: 5 days with activated-charcoal, 23 days lacking activated charcoal	84	53

Under the three condition tested, rooting efficiencies of shoots-tip explants harvested from shoot masses produced in liquid KM medium were higher than for shoot-tip explants harvested from shoots previously cultured on solid KM micropropagation medium lacking activated charcoal. Rooting of shoots-tip explants harvested from the fermenter was first observed after 10

days transfer to solid rooting medium, two days earlier than shoot-tips harvested from shoots previously cultured on solid KM micropropagation medium lacking activated charcoal.

The micropropagation of E. grandis described above gave high multiplication rates and yielded an abundance of shoots for subsequent rooting. The shoots for rooting showed an increase of about 50 fold per month and the total number of explants that could be used for any subsequent micropropagation steps increased in excess of 100-fold per month. The high levels of multiplication were mirrored by the increase in biomass.

As described above, the quality of the shoots harvested from the shoot masses produced in liquid media was high, with few overt signs of hyperhydricity. The signs of hyperhydricity that were observed were confined to older tissues. The shoots harvested from the fermenters appear to root more readily on solid media containing IBA than control shoots harvested directly from cultures propagated on solid medium. The quality of the shoots is such that they may be suitable for direct rooting into compost in the greenhouse, as with the shoots of Acacia mangium described in Example 11 below.

EXAMPLE 2

Propagation of E. grandis hybrid clones & E. grandis seedlings

A number different E. grandis hybrid clones and the micropropagated E. grandis seedling line T14 L10 were micro-propagated using the air-lift method described in Example 1 except that commercially available polycarbonate vessels having a capacity of about 2.5 litres ("2 litre" vessels of Nalgene, Nalge Co. Box 20365, Rochester, NY 14602-00365, USA; cat. no. 2015-2000) and polypropylene screw tops containing inlet and

outlet ports (Nalgene cat. no. 2162-0531) were used instead of the glass vessels.

The oxygenation/aeration system was fitted using a preformed (inlet) port as described in Example 1. 2.0 litre of liquid KM micropropagation medium as described above was placed in each vessel and the vessels were autoclaved (10 min preheat 20 min full pressure (12 °C) cycle). Once cooled, each vessel was connected to the air supply for equilibration for 1 hour prior to inoculation with 20 shoots of one of the clones or of the seedling line indicated, which clones and seedlings had previously been propagated on solid KM medium. Operation of the vessels was terminated after 22 days because some of the vessels had become full of propagating shoot masses.

Five typical shoot masses from each vessel were dissected in order to estimate the multiplication rates achieved. Table 4 show the multiplication rates obtained for the five clones and one seedling line used.

TABLE 4

Multiplication rates of different E. grandis genotypes and hybrids in air-lift fermenters

Shoot genotype	Multiplication rate (after 22 days) (av. of 5 shoots)
Seedling line T14 L10	24.4
E. grandis x camaldulensis clone 11/25	24.7
E. urophylla x grandis clone 18.50	31.3
E. urophylla x grandis clone 18.52	30.7
E. grandis clone 5046	11.7
E. grandis clone 5048	3.1

For five of the six genotypes used, the multiplication rates are considerably higher than could be achieved using a semi-solid (gelled) medium system. In four of the six genotypes, multiplication rates are 8 to 10 times higher than could be achieved using a semi-solid (gelled) medium system.

EXAMPLE 3

Propagation of eucalyptus shoots using an airlift system with restrained shoots

The airlift system used in Examples 1 and 2 was modified by restraining the inoculated shoots in a 125 cm^3 stainless steel cage containing perforations to allow the free passage of medium over the shoots instead of allowing the shoots to move freely in the liquid medium. A duplicate was carried out in which the shoots

were unrestrained.

The method used was as described in Example 2 except that 250 ml polycarbonate containers (Nalgene cat. No. 2127-0250) and polypropylene screw tops containing inlet and outlet ports (Nalgene cat. no. 2162-0531) were used instead of the vessels previously described. The volume of medium used was 150 ml per vessel. Preparation and operation of the vessels was as described for the previous example.

Each vessel was inoculated with either 5 shoots of E. grandis x camaldulensis clone 11/25 or with 5 shoots of the micropropagated E. grandis seedling line T14 L10, the shoots having previously been propagated on solid KM medium cultures. In one set of vessels the shoots were allowed to move freely. Another set of vessels the shoots were restrained in a cage as described above. The shoots were cultured for 21 days then dissected to determine the multiplication rates achieved, which are shown in Table 5 below:

TABLE 5

Shoot genotype	Multiplication rate (after 21 days) (av. of 5 shoots)	
	Unrestrained	Restrained
E. grandis x camaldulensis clone 11/25	8	10
E. grandis seedling line T14 L10	17.7	16.8

The multiplication rates achieved indicate that free movement of the shoots is not necessary to achieve high

multiplication rates.

EXAMPLE 4

Propagation of eucalyptus shoots in a shaken flask system.

The method used was as described in Example 3 except that the gas diffusion tube was not present in the apparatus. The vessels were vented to the atmosphere using a single filter attached to an inlet/outlet port as previously described. The vessels were shaken at 125 r.p.m. on an Infors CH1043 shaker for 22 days. The shoots used were from the micropropagated seedling line T14 L10. Duplicate sets of vessels were run. In one set the shoots were not restrained; in the other they were restrained in a cage as described in Example 3.

The multiplication rates obtained were 2 for the unrestrained shoots and 4.2 for the restrained shoots. This result demonstrates that multiplication will occur in a shaken flask in which plant material is fully submerged.

EXAMPLE 5

Propagation of eucalyptus using a stirred system

The procedure described in Example 4 was carried out except that, instead of being shaken, the contents of the vessels were stirred using a 2.5 cm magnetic stirrer bar. The vessels were placed on magnetic stirrers adjusted to give a rotation rate of the stirrer bar of approximately 250 r.p.m. Each vessel was inoculated with either 5 shoots of E. grandis x camaldulensis clone 11/25 or with 5 shoots of the micropropagated E. grandis seedling line T14 L10. Duplicate sets of vessels were run; in one set the shoots were restrained in a cage as described in Example 3, in the other set the shoots were allowed to move freely. The results are given in Table 6 below.

TABLE 6

Shoot genotype	Multiplication rate (after 22 days) (av of 5 shoot masses)	
	Unrestrained	Restrained
E. grandis x camaldulensis clone 11/25	macerated	8
E. grandis seedling line T14 L10	macerated	3.2

No usable shoots were recovered from the vessel containing the unrestrained shoots due to mechanical damage of the shoots caused by the magnetic stirrer bar.

EXAMPLE 6

Propagation of eucalyptus shoots in the light & in the dark

The procedure described in Example 2 i.e. using the airlift system was repeated except that 250 ml vessels were used and the shoots were from the micropropagated E. grandis seedling line T14 L10. The lighting conditions were as described at the beginning of this section i.e. 16 hour photoperiod light (50-70 µmol m^{-2} s^{-1} supplied by fluorescent lamps). A duplicate was carried out, but in continuous darkness instead of continuous light.

The multiplication rates obtained after 22 days culture were 24.4 for the vessel incubated in the light and 21.2 for the vessel incubated in the dark. This indicates that light is not essential in order to obtain high shoot multiplication rates provided oxygenation is adequate.

The shoots cultured in the dark were more elongated

than those produced in the light, indicating that manipulation of lighting conditions can be used to manipulate shoot quality, which is important in commercial micropropagation systems. For example, more elongated shoots of E. grandis and related hybrids often root more efficiently than less elongated shoots.

EXAMPLE 7

Propagation of eucalyptus with oxygenation by photosynthesis

The procedure described in Example 6 i.e. cultivation in the light and in the dark was carried out except that the gas distribution tubes were omitted from the apparatus. In this system there is no agitation other than by convection and/or diffusion, and no active i.e. externally applied oxygenation. The shoots were from the micropropagated E. grandis seedling line T14 L10.

The shoot multiplication rates obtained after 22 days were 6.2 in the vessel incubated in the light and 1 (no multiplication) in the dark. The result indicates that photosynthesis can compensate at least in part for the lack of active oxygenation i.e. externally applied oxygenation.

EXAMPLE 8

Propagation of eucalyptus with oxygenation and light

The method described in Example 6 using the continuous light conditions was repeated except that the air bubbled through the liquid medium was replaced by pure nitrogen. Shoots of E. grandis x camaldulensis clone 11/25 were used.

A 4-fold multiplication rate was obtained using the nitrogen, as compared to a multiplication rate of 10 when air is used (see Example 6). This indicates that adequate oxygenation, by active i.e. externally applied oxygenation and/or by photosynthesis, is required to support the multiplication of the shoots.

EXAMPLE 9

Propagation of eucalyptus using different inoculum sizes

The method described in Example 2 was carried out except that the vessels were inoculated with either 5, 20 or 40 shoots of E. grandis x camaldulensis clone 11/25. After 23 days incubation, multiplication rates obtained were 8, 24.7 and 36.4, respectively. This suggests that there is an advantage in using a high inoculation density.

EXAMPLE 10

Propagation of eucalyptus by recycling shoot material

Using the procedure described in Example 2, E. grandis x camaldulensis clone 11/25 and micropropagated seedling line T14 L10 were inoculated into the culture vessels and harvested after 23 days. 20 shoots were dissected from the shoot masses and immediately inoculated into vessels containing fresh liquid medium and incubated for a further 23 days. This process was repeated for one more cycle, giving a total of three propagation cycles.

The shoot multiplication rates did not differ significantly between cycles and there was no deterioration in the quality of the shoots produced after successive cycles. This demonstrates that shoot material can be recycled through successive rounds of liquid micropropagation, which is a significant commercial advantage.

EXAMPLE 11

Propagation of Acacia mangium

(i) Micropropagated cultures of Acacia mangium were established from seed (seedlot 945) obtained from HDRC, Ryton on Dunsmore, Coventry CV8 3LG UK.

(ii) Micropropagated Acacia mangium shoots previously produced on semi-solid medium comprising Complete MS

salts Medium (Murashige and Skoog, 1962) supplemented with 2.2 μM BAP were inoculated into liquid medium of the same composition but lacking gelling agent. The method and apparatus were otherwise as described in Example 2.

The shoots do not show signs of hyperhydricity (vitrification) and multiply well (multiplication rates of 12-15 fold/month). Shoot clumps form roots from the base. The shoot multiplication rate of this seedling-derived material using the method previously described on semi-solid medium is less 3 fold/month.

(iii) 10-20 mm long shoots produced using the liquid micropropagation described in section (ii) above were harvested, dipped in rooting powder (Seradix No. 2 rooting powder, Hortichem Ltd. Salisbury, Wilts SP2 7NU, UK) and set for rooting in compost in a fog chamber (96% relative humidity) at 25 °C under shaded daylight (maximum 250 μmol m^{-2} s^{-1}). Rooting efficiencies obtained were greater than 70% for material produced in the process compared with less than 60% rooting for shoots produced on semi-solid medium using the same rooting procedure.

(iv) The effect of repeated cycling of Acacia mangium shoot material through successive rounds of liquid micropropagation was determined using the method described in section (ii) above. Shoots were harvested after an initial cycle of one month propagation in the air lift fermenter system described in section (ii) above, harvested and reinoculated into vessels containing fresh medium and cultured for a further month. This process was repeated for one more cycle, giving a total of three propagation cycles. The shoot multiplication rates did not differ significantly between cycles and there was no noticeable deterioration of the quality of the shoots produced after repeated cycling. The ability to recycle the starting material is a considerable

commercial advantage.

EXAMPLE 12

Propagation of Olive

Micropropagated shoots of Olea europaea cultivar Dolce Agogia previously produced on semi-solid medium as described by Rugini et al. (Sci Hortic (Amst), 24(2), 1984, 123-134) were inoculated into liquid medium of the same composition but with the phytohormones adjusted to 4.9 μM Zeatin and 2.9μM GA_3 (gibberellic acid) and lacking gelling agent. The method and apparatus were otherwise as described in Example 2.

Very healthy material was obtained. Multiplication rates are slightly higher than any published for gelled media systems (7 fold/month in the liquid system vs a maximum of 6/month for this genotype on gelled media systems) and there were two further, important advantages of the liquid system over the gelled system:

1) The resulting shoots are much healthier; gelled systems all report chlorosis (yellowing) whereas the liquid system produces dark green, healthy shoots.

2) The shoots produced in the liquid system are more elongated and therefore do not require an extensive elongation step prior to rooting. Shoots produced on gelled media systems require a lengthy (more than 1 month) elongation step prior to rooting.

EXAMPLE 13

Propagation of Rhododendron

Micropropagated shoots of Rhododendron yakushimanum cultivar Dopey previously produced on semi-solid medium as described by Lloyd and McCowan (Combined Proceedings of the International Plant Breeders Society 30, 1980, 421-437) were inoculated into liquid medium of the same composition but with the phyto-hormones adjusted to 2.5 μM 2iP (N^6-(2-isopentyl adenine) and lacking gelling agent. The method and apparatus were otherwise as

described in Example 2.

6-fold multiplication was obtained in 4 weeks, compared with 2-3 fold multiplication in 6 weeks on gelled medium.

The uniformity of the shoots obtained according to the liquid culture system was excellent and far superior to that obtained using gelled propagation systems. One major advantage of the liquid system is that no adventitious shoots are produced and all new shoots are produced from axillary buds. In all the gelled systems, large amounts of callus can be formed on the base of the multiplying shoots. Adventitious shoots are often produced from this callus via organogenesis. These adventitious shoots are sometimes not true to type (i.e. are abnormal) due to the phenomenon of somaclonal variation. (This phenomenon is often associated with changes in chromosome number or chromosomal rearrangements.) Currently, commercial Rhododendron producers have to sacrifice multiplication rates in order to avoid callus formation and adventitious shoot formation to ensure that the plants they are propagating are true to type. The liquid system of the present invention avoids this problem.

Shoots produced in the bioreactor were elongated by 2-3 weeks culture on the semi-solid medium as described above prior to rooting into compost using the method as described for Acacia mangium. Rooting efficiencies obtained were in excess of 90% and equivalent to rooting efficiencies of shoots produced on semi-solid medium as previously described, using the same rooting procedure. Manipulation of the growth conditions in the bioreactor will facilitate the production of more elongated shoots which will be suitable for setting for rooting immediately after harvest from liquid culture.

EXAMPLE 14

Propagation of Malus domestica

Micropropagated Malus domestica cultivar Greensleeves shoots previously produced on semi-solid medium comprising complete MS salts Medium (Murashige and Skoog, 1962) supplemented with 5 μM BAP, 0.5 μM IBA and 3μM GA_3 were inoculated into liquid medium of the same composition but lacking both gelling agent and plant growth regulators, the method and apparatus otherwise being described in Example 2.

Shoot multiplication rates achieved were 3/month, the same as that obtained on semi-solid medium described above. However, the liquid system requires fewer handling steps than the semi-solid system, so has commercial advantages. The shoots obtained showed some symptoms of hyperhydricity but when shoots harvested from the vessel were transferred to semi-solid medium as described above, all new growth was free of symptoms.

EXAMPLE 15

Propagation of Forsythia

15 micropropagated shoots of Forsythia x intermedia cv. Lynwood previously produced on semi-solid medium LS medium (Linsmaier ES & Scoog S, Phys. Pl. (1965) 18 100-127) supplemented with 3% sucrose and 10 μM BAP were inoculated into liquid medium of the same composition but lacking gelling agent, the method and apparatus otherwise being described in Example 2. The shoots were harvested after four weeks.

The harvested shoots had grown well. There were no signs of hyperhydricity and the shoots were similar in appearance, in particular in texture and colour, to shoots grown on the semi-solid medium. However, on the semi-solid medium a clump of many short shoots may develop, of which only 2-3 shoots normally elongate. In the liquid medium all the shoots had elongated,

indicating that overall multiplication rates may be considerably higher than on the semi-solid medium. The shoots harvested from the liquid medium appeared to be suitable for rooting directly into compost under fog in a greenhouse.

EXAMPLE 16

Propagation of Syringia

Semi-solid LS medium (Linsmaier ES & Scoog S, Phys. Pl. (1965) 18 100-127) supplemented with 3% sucrose and 30 μM BAP or with 3% sucrose and 10μM zeatin were used for the micropropagation of shoots of Syringia vulgaris cv. Madame Lamoine. The BAP-supplemented medium gives good proliferation but poor elongation while the zeatin medium gives poor shoot proliferation. Clumps of shoots produced on the BAP medium were transferred to the zeatin medium for elongation. For liquid culture the BAP medium without the gelling agent was used as the liquid medium.

15 Micropropagated shoots were inoculated into the liquid medium, the method and apparatus otherwise being described in Example 2. The shoots were harvested after four weeks. The original explant leaves had turned brown but new growth was dark green and showed few symptoms of hyperhydricity. The shoots appear to be suitable for rooting directly into compost under fog in a greenhouse.

EXAMPLE 17

TRANSFORMATION, SELECTION AND PROPAGATION OF E. GRANDIS CLONES, E. GRANDIS/E. CAMALDULENSIS HYBRID CLONES AND E. SALIGNA/E. TERETICORNIS HYBRID CLONES

a) Disarmed Agrobacterium strain

The construction of A. tumefaciens strain EHA101 has been described by Hood et al., 1986. The strain consists of a derivative of the of nopaline A. tumefaciens strain C58 in which the native Ti plasmid has been removed and replaced with the disarmed Ti plasmid pEHA101 in which the wild-type T-DNA (ie opine synthesis

and phytohormone genes) has been deleted from the Ti plasmid and replaced with a bacterially-expressed kanamycin/neomycin resistance gene. The disarmed plasmid pEHA101 is a derivative of the wild-type Ti plasmid pTiBo542 isolated from A. tumefaciens strain Bo542 (AT4) which is a L,L-succinamopine producing strain (Hood et al., 1986). Strain EHA101A is a chloramphenicol resistant mutant of strain EHA101 which was isolated by Olszwelski et al., 1988.

b) Binary vector construct

The strain used in the transformation also contains the binary Ti plasmid pSCV1.6, which is a derivative of pSCV1. Genetic manipulations involving these plasmids were performed using standard techniques (Sambrook et al., 1989). The component parts of pSCV1 are derived from the following (gram-negative) plasmids: the sequence used for the right DNA border and overdrive sequence was synthesised using sequence information from from the TL right border of the octopine Ti plasmid pTiA6 (Peralta et al., 1986). The left border was synthesised using sequence information from the TL of the same Ti-plasmid (Simpson et al., 1982) and is identical to the TL left border of the octopine plasmid pTiACH5 (Holsters et al., 1983). Octopine-type border sequences were used as these have been shown to promote more efficient tumour formation when used in conjunction with the hypervirulent strain EHA101 (Hood et al., 1986). The 97bp polylinker containing restriction enzyme sites for cloning genes into the T-DNA was derived from pUC19 (Yannish-Perron et al., 1985). The high copy number origin of replication which is active in E. coli cells but not Agro-bacterium cells was derived from pUC19 (Yannish-Perron et al., 1985). The origin of replication of pUC 19 which was itself originally derived from the plasmid ColE1, a plasmid isolated from E. coli. The actual pUC sequence

used has been extensively deleted to remove some non-functional (superfluous) DNA sequences. The low copy number origin of replication which is active in both E. coli cells and Agrobacterium cells was derived from the the broad host-range Inc P plasmid RK2. The origin used is a minimal 4.3kb origin which was constructed by deleting most of the non-functional sequences originally present in the wild-type RK2 plasmid (Thomas et al., 1980). The minimal origin therefore contains only two genes (trf A and trf B) and associated non-coding sequences needed for replication in bacteria. The bacterially-expressed gentamicin/kanamycin resistance gene was derived from the plasmid pSa (Edwards, 1988) and is probably an aminoglycoside acetylase (Valantine and Kato, 1989). It has no apparent homology to the neomycin phosphotransferase II coding region (Edwards, 1988). The bacterially-expressed ampicillin/carbenicillin resistance (ß-lactamase, bla) gene was cloned from pUC19 (Yannish-Perron et al., 1985). A genetic and restriction map of pSCV1 is shown in Figure 3.

In Figure 3 Amp^R and Gm/Km^R denote antibiotic resistance genes for plasmid selection in bacteria. trfA, trfB, RK2 and Col E1 origins denote baterial replication functions. OD denotes an overdrive (T-DNA transfer enhancer) sequence. Bam H1, Bcl 1, Cla 1 etc denote restriction endonuclease recognition sequences. Map units are given in Kilo base pairs of nucleotide sequence.

pSCV1.6 is a derivative of pSCV1, into which a plant-expressed ß-glucuronidase (GUS) gene and a plant-expressed kanamycin resistance gene were cloned between the T-DNA borders. The CaMV-NPTII was derived from the construct of Fromm et al., 1986. However, it has been reported that several of the most common NPTII genes used in plant genetic-manipulation encode a mutant enzyme that

has a reduced ability to detoxify kanamycin (Yenofsky et al., 1990). The mutation involves a single base change resulting in the replacement of a glutamic acid residue by an aspartic acid at the active site of the neomycin phosphotransferase (NPTII) enzyme (originally isolated from the bacterial transposon Tn5). While the stability of the mRNA and the protein appeared unaffected by the mutation, the enzyme activity towards kanamycin is significantly reduced. The presence of the mutation in a gene can be identified by checking for the loss of a site for the restriction endonuclease XhoII in the NPTII coding sequence. This mutation was found to be present in the CaMV-NPTII gene of Fromm et al., 1986 and was repaired in the following manner. The plasmid pSUP2021 (Simon et al, 1983) is approximately 10kb in size and includes a complete copy of the transposon Tn5. Digestion of this plasmid with Pst 1 and Sma 1 gives a 788 bp fragment that extends from position 1730 to 2518 within Tn5 (Beck et al., 1982). This fragment was isolated and restricted with Sph 1 (giving fragments of 352 and 436 bp) or XhoII (giving fragments of 120, 246, 394 and 28 bp), and is therefore "wild-type" with respect to the mutation at position 2096. The Pst 1/Sma 1 fragment was subcloned into Pst 1/Sma 1 cut pUC19 to give pTn5sub. This was then digested with Sma 1 and ligated with 8 mer phosphorylated Bam H1 linkers. A clone in which the Sma 1 site had been converted to a Bam H1 site (pTn5subA) was then digested with Sph 1 and Bam H1 and the 436 bp fragment (from position 2082 to 2518) isolated. This was used in a tripartite ligation with the 542 bp Bam H1/Sph 1 fragment from pCaMVNeo (positions 1540 to 2082) and Bam H1 digested pUC19. Recombinants were restricted with Bam H1 and Sph 1 to ensure that they contained both the 436 and 542 Bam H1/Sph 1 fragments, and Xho II to confirm that the site at position 2096 had been restored. This

construct has a Bam H1 fragment which contains the NPTII gene coding sequence which is essentially identical to the Bam H1 fragment used by Fromm et al., (1986) to make pCaMVNeo, except that the mutation has been corrected. This construct was designated pneoNeo. The Bam H1 insert of pneoNeo containing the NPT11 coding sequence was then isolated and religated with the large (approx. 3 kb) fragment isolated from Bam H1 restricted pCaMVNeo, this fragment containing the vector plus CaMV promoter and nopaline synthase gene 3' termination sequence. Recombinants were checked against pCaMVNeo for the correct orientation using both Pvu II (2 sites) or Eco R1/Sph 1 (both unique), giving pCaMVneoNeo. This was again checked for the correct number of Xho II sites.

The Hind III fragment from pCaMVneoNeo containing the restored plant-expressed kanamycin resistance gene was cloned into the Hind III site of pSCV1 to give the plasmid pSCV1.2. pSCV1.2 was partially digested with HindIII and the linear 10.2 kb product isolated. This was dephosphorylated with calf intestinal alkaline phosphatase and ligated with a 2.8 kb Hind III DNA fragment containing a plant expressed ß-glucuronidase gene (CaMV-GUS INT gene) isolated from the plasmid pGUS INT which has been described by Vancanneyt et al., 1990.

A map of the T-DNA in the resultant construct (pSCV1.6), indicating the orientation of the genes and the region of DNA for transfer to plants are shown in Figure 4.

In Figure 4 the abbreviations given in the map have the following meanings: B = Bam H1; Bg = Bgl II; C = Cla 1; E = Eco R1; EV = Eco RV; H = Hind III; K = Kpn 1; P = Pst 1; S = Sac 1; Sm = Sma 1; Sp = Sph 1; X = Xba 1; Xh = Xho 1; OD = Over-drive (T-DNA transfer enhancer)

c) Introduction of the binary plasmid vector pSCV1.6 into the disarmed A. tumefaciens strain

Cells of Agrobacterium tumefaciens strain EHA101A were transformed by electroporation using a Biorad Gene Pulser as described by Wen-jun and Forde (1989).

d) Preparation of Agrobacterium inoculum

Overnight liquid cultures of Agrobacterium tumefaciens strain EHA101A containing the binary plasmid pSCV1.6 were grown on YEB medium (tryptone 5 g l^{-1}, yeast extract 1 g l^{-1}, beef extract 5 g l^{-1}, magnesium sulphate 0.46 g l^{-1}, pH 7.2 and sucrose 5 g l^{-1} added after autoclaving) containing 50 mg l^{-1} chloramphenicol, 25 mg l^{-1} neomycin and 15 mg l^{-1} gentamicin at 28 °C with vigorous shaking. 10 µl of a fresh overnight liquid culture was inoculated into 25 ml of fresh media and grown for 24 h. The cells were harvested by centrifugation at 6000 g for 10 minutes, resuspended in 2mM $MgSO_4$ and repelletted. The cells were washed once more in 2mM $MgSO_4$ and once in liquid clone co-cultivation medium (see later). The cells were finally resuspended in liquid clone co-cultivation medium and diluted to a density of 10^9 cells ml^{-1} ready for co-cultivation with the explants.

e) Plant material

E. grandis clone 91/4 and E. grandis/E. camaldulensis hybrid clone 11/25 were supplied by the South African Forestry Research Institute, PO Box 727, Pretoria 0001, Republic of South Africa (now FORESTEK, Private Bag X11227, Nelspruit 1200, South Africa). E. saligna/E. tereticornis hybrid 2.32 was obtained from Centre de Development Forestier, B.P. 764, Pointe Noire, Republique Du Congo. Stock plants were obtained by felling mature trees and harvesting cuttings from new growth arising from from epicormic buds in the stump. Cuttings were rooted using routine silvicultural techniques and

subsequently potted into 10 litre pots and maintained in the glasshouse as hedged stockplants. Where required, *in vitro* micropropagated shoot cultures were initiated from these stockplants by harvesting nodal stem explants from stockplants and disinfecting by immersion in a 20% v/v Milton solution containing 0.1% v/v Tween 20 for 10 minutes with gentle agitation. The nodal stem explants were then briefly rinsed three times in sterile distilled water and cultured on shoot multiplication medium (190 mg l^{-1} KNO_3, 825 mg l^{-1} NH_4NO_3, 220 mg l^{-1} $CaCl_2.H_2O$, 925 mg l^{-1} $MgSO_4$, 85 mg l^{-1} KH_2PO_4, half-strength Murashige and Skoog basal salt micronutrient solution (catalogue number M0529), vitamins as described by Morel and Wetmore (1951), 10 g l^{-1} sucrose, 0.04 mg l^{-1} BAP, 300 mg l^{-1} augmentin, pH adjusted to 5.6 with KOH, 2 g l^{-1} phytagel). The cultures were propagated at 23 ^{O}C using a 16 hour day illumination regime (50-70 µmol m^{-2} s^{-1}). The multiplying shoots were divided and subcultured onto fresh clonal shoot multiplication medium at 4 weekly intervals.

f) Preparation of explants for transformation

Leaf, petiole or stem explants from the clones were prepared directly from axenic micropropagated shoot cultures or rooted micropropagated shoots without disinfection (protocols for micropropagation and susequent rooting of shoots are given below). Alternatively, leaf, petiole or stem explants were prepared from ramets (either produced via micropropagation or by cuttings) grown in the greenhouse or in the field and disinfected prior to co-cultivation with *Agrobacterium tumefaciens*. In this case, young scions with healthy leaves less than 3 cm in length were harvested from the upper portion of the crown from vigorous plants of less than 1.5 metres in height, and disinfected by immersion in a 20% v/v Milton solution

containing 0.1% v/v Tween 20 for 10 minutes with gentle agitation. The scions were then rinsed three times in sterile distilled water prior to dissection. 3-5 mm diameter leaf explants or 2-4 mm long sections of stem or petioles were prepared from the scions and placed in liquid clonal co-cultivation medium (see below) until required for co-cultivation with the A. tumefaciens strain.

g) Inoculation of explants with Agrobacterium and regeneration of putative transgenic shoots

Leaf, petiole or stem explants of the clones previously described were co-cultivated with the Agrobacterium suspension, prepared as described previously, for 15 minutes in a sterile 9 cm petri dish. The dish was placed on an orbital shaker and gently shaken at 23 °C during the incubation. After incubation, excess bacterial suspension was removed from the explants by blotting with filter papers and the hypocotyl explants were transferred to solid clone co-cultivation medium (750 mg l^{-1} KNO_3, 250mg l^{-1} $MgSO_4.7H_2O$, 250 mg l^{-1} $NH_4H_2PO_4$, 100mg l^{-1} $CaCl_2.2H_2O$, 20 g l^{-1} sucrose, 600 mg l^{-1} 2-[N-morpholino]ethanesulphonic acid (MES), half-strength Murashige and Skoog basal salt micronutrient solution (Sigma catalogue number M0529), vitamins as described by Morel and Wetmore (1951), 0.1 to 1 (eg 1) mg l^{-1} CPPU, 0.465 mg l^{-1} NAA, pH adjusted to pH 5.5 with KOH, 3 g l^{-1} phytagel). The explants were co-cultivated with the Agrobacterium strain for 48 h in the dark at 23 °C. After incubation, excess bacterial suspension was removed from the explants by blotting with filter paper and the explants were then washed twice (3 hours per wash) in liquid clone co-cultivation medium containing 400 mg l^{-1} augmentin at 23 °C with gentle shaking. The explants were then transferred to clonal shoot induction medium (as for clone co-cultivation

medium but containing 500 mg l^{-1} glutamine, 50 mg l^{-1} ascorbic acid and 300 mg l^{-1} augmentin. The explants were incubated in the dark at 23 °C for 4 weeks with subculture to fresh medium after 2 weeks and at the end of the period of incubation in the dark. The cultures were then transferred to continuous light (40 µmol m^{-2} s^{-1}) and incubated at 23 °C. The cultures were then subcultured every two weeks onto fresh clonal shoot induction medium until significant numbers of shoot primordia were visible. The explants were subcultured onto clonal shoot elongation medium (as clonal shoot induction medium) but with the CPPU ommitted, the NAA concentration adjusted to 0.112 mg l^{-1} and containing 1.16 mg l^{-1} BAP and incubated at 23 °C under continuous light (40 µmol $m^{-2}s^{-1}$).

h) <u>Selection, multiplication and rooting of putative genetically modified shoots</u>

When suitable numbers of regenerating shoots more than about 1mm long are present on the explants, the explants were transferred to an air-lift fermenter containing the liquid KM micropropagation medium described above, 300 mg l^{-1} augmentin and 30-120 mg l^{-1} paromomycin, generally 50-60 mg l^{-1}. After a suitable period, generally 5-20 days depending on the concentration of paromomycin used, putative genetically modified shoots are identified by their healthy green appearance and rapid growth and extension in comparison to the majority of non-GM shoots which become brown and necrotic. The putative genetically modified shoots were then transferred to fresh liquid KM micropropagation medium (with or without paromomycin) and multiplied for a further 1 month, each individual original shoot now forming a large mass of branching shoots. These shoots were then rooted by transfer to rooting medium (as clonal shoot multiplication medium i.e. the solid KM medium but

with the BAP omitted and containing 0.2 mg l^{-1} IBA) and incubation for 24h at 23 °C using 16 hour day illumination regime of 50-70 µmol $m^{-1}s^{-1}$. Additional multiplication steps, either in liquid culture or on solid culture may be conducted as required prior to rooting of shoots. Following the root-induction step, shoots with developing roots were transferred to a sterile peat pellet (Jiffy Products (UK) Limited, 14/16 Commercial Road, March, Cambridge, UK) in a Magenta pot (Sigma) for root establishment.

When actively growing roots were visible growing through the peat pellet, the plant was transferred to an approximately 7.5 cm (3 inch) square plant pot filled with coco-peat. The plants were placed inside a mist propagator and slowly hardened off by reducing the humidity over a period of a week. After three to four weeks, the plants were transferred to approximately 17.5 cm (7 inch) pots and placed in a glasshouse facility. The plants were grown under natural daylight and were watered daily.

i) The advantageous results observed in Example 1 for plants produced by the liquid micropropagation system were also observed in the present case for the genetically modified plants that were selected and micropropagated using the air-lift fermenter.

In addition, very considerable savings in time, labour and materials were achieved in comparison with the conventional method of selection and micropropagation on solid (gelled) media and cocultivation are common to both the conventional process and that of the present invention.

The initial steps in the Agrobacterium-mediated transformation are common to both methods. The conventional methods then combine a shoot induction step with a selection step, using a solid medium. As

discussed above, the presence of the selective agent often slows the growth and/or development of the transformed cells and tissues. This is illustrated in the present case, where selection of genetically modified Eucalyptus clones on solid media using G-418 as the selective agent takes about 21 weeks. The subsequent step of shoot elongation of the selected genetically modified shoots on solid media takes about 6 weeks, making a total of about 27 weeks to obtain material suitable for micropropagation.

In contrast, in the present Example, the putative genetically modified material is cultured initially on a solid medium without selection, for about 9 weeks. Selection is then carried out in submerged liquid culture. Transformed material can be distinguished from non-transformed material within as little as 10 days. The total time for the selection step is therefore just over 10 weeks, in comparison with the 27 weeks required for selection using convention methods. Not only is the time reduced dramatically, with very considerable savings in labour and materials, the selected material is of particularly high quality, having the properties and characteristics described in Example 1 for the micropropagated shoots that had not been subjected to transformation. The shoots are green and healthy, having shoot tips that are well elongated, posses thick and robust stems and well spaced nodes. The shoots are remarkably uniform, which is particularly important commercially.

The subsequent micropropagation of the transformed Eucalyptus shoots takes about 16 weeks by a conventional method using a solid medium. Using the high quality material obtained after the submerged liquid selection step, micropropagation using the submerged liquid culture method takes about 6 weeks, giving a further time saving

(of ten weeks) and also yielding material of particularly high quality, as described above and in Example 1.. The shoots obtained by the submerged liquid culture appear to root more readily than those from conventional solid culture.

In summary, the method described in this Example results in savings in time, labour and materials and yields a greater quantity of transformed material that is of higher quality than that resulting from conventional methods.

BIOCHEMICAL AND GENETIC ANALYSIS OF THE GENETICALLY MODIFIED EUCALYPTUS PLANTS

i) Histochemical ß-glucuronidase (GUS) assays

Histochemical GUS assays were performed on the leaves of putative genetically modified Eucalyptus clones and seedling-derived material as described by Draper et al. (1988). Leaf explants were transferred to a petri dish containing fixation solution (100 ml double distilled water containing 750 µl 40% formaldehyde, 2 ml 0.5 M MES and 5.46 g l^{-1} Mannitol). The petri dish was placed in a vacuum desiccator and the vessel was evacuated several times until all of the explants were submerged in the fixation solution. The explants were incubated for 45 minutes at room temperature and then washed twice in 50mM sodium phosphate buffer (pH 7.0). The explants were then transferred into a 2mM 5-bromo-4-chloro-3-indoyl glucuronide (X-GLUC) solution made up in 50mM sodium phosphate buffer (pH 7.0). The X-GLUC solution was vacuum infiltrated into the explants several times, the dish sealed with Nescofilm and then incubated at 37 °C overnight. The reaction was stopped by transferring the explants to 70% ethanol. GUS activity could be detected by the presence of an insoluble blue stain.

ii) Detection of genes transfered to transgenic Eucalyptus plants by Southern blotting and hybridisation

DNA extraction was carried out as described by Keil and Griffin (1994). 10 micrograms of DNA isolated from transformed Eucalyptus plants were digested with with Kpn1 and Xba1 in the appropriate restriction buffers. To aid the digestion of DNA, casein was added to the restriction mixture at a final concentration of 0.1 mg/ml (Drayer and Schulte-Holthausen, 1991). The restrictions were carried out at 37 °C overnight. Electrophoresis of the samples, Southern blotting and hybridisation were performed as described by Sambrook et al. (1989). The plasmid pJIT65 (Guerineau, 1990) was digested with Eco RV and the plasmid pCaMV digested with Bam H1. The resulting restriction fragments were separated by electrophoresis on a 1.5% agarose gel (Sambrook et al., 1989). A 2kb (approximately) DNA fragment containing part of the coding sequence of the GUS gene and the Cauliflower Mosaic Virus 35S gene terminator region and a 1.0 kb (approximately) DNA fragment containing the NPT2 coding sequence were eluted from the gel by the method of Heery et al. (1990). The eluted fragments were radiolabelled by the method of Feinberg and Vogelstein (1983), using the random primer labelling kit supplied by Boehringer Manheim and used as hybridisation probes.

iii) Results

The process of the invention as described in sections a) to h) aboveset out above enabled transformed Eucalyptus plants to be produced efficiently and in short periods of time, even from explants originating from mature plants (clones) which had previously been grown in the field and for which production of transformed plants has not proved possible. The efficiency of these methods enabled large populations of plants each resulting from individual transformation events to be produced from any

one of the Eucalyptus species or hybrids transformed. In all of the examples, genetically modified shoots were obtained via organogenesis from genetically modified callus. In some cases, mixed organogenesis and somatic embryogenisis could be observed in some of the cultures, particulary if culture periods on regeneration media were continued for extended periods. In all of the methods described, viable plants were recovered that exhibited normal phenotypes when grown under greenhouse conditions. A high proportion (in excess of 70%) of the genetically modified plants from any one of the Eucalyptus species or hybrid transformed were found to express the ß-glucuronidase gene as determined by histochemical staining. Similarly, at least 80% of the regenerated shoots were found to contain at least one of the genes from the T-DNA of pSCV1.6 integrated into the genome of the Eucalyptus species or hybrid.

<u>Analysis of genetically manipulated Eucalyptus plants using the polymerase chain reaction (PCR) for the presence of the T-DNA and for absence of the Agrobacterium tumefaciens strain used in their production</u>

<u>Method</u>:

PCR reactions were conducted using a Perkin-Elmer Cetus DNA Thermal Cycler (Perkin-Elmer, Beaconsfield, Bucks. UK). The reaction consisted of 1x reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% w/v gelatin), 200 µM each dNTP, 1.0 µM of each primer, 2.5 units of Amplitaq DNA polymerase (Perkin-Elmer) and 0.5 µg genomic DNA isolated from genetically manipulated shoots as previously described. Control reactions containing genomic DNA from plants known to be free of <u>Agrobacterium tumefaciens</u> or containing approximately 10 ng DNA isolated from <u>Agrobacterium tumefaciens</u> EHA101A [pEHA101, pSCV1.6] were also conducted. Reaction conditions used were 29 cycles of 1 min at 94 °C, 1 min

annealing and 1 min at 72 °C and one cycle of 1 min at 94 °C, 1 min annealing and 2 min at 72 °C. Samples of each reaction were electrophoresed on a 2% agarose gel and visualised under UV light. Primers for the detection of the following gene sequences and the annealing temperatures used in the reactions were as described below:

NPTII gene (using the TN5 numbering system described by Beck et al., (1982) Gene 19, 327-336).

PRIMER 1: 5' (24) CGCAGGTTCTCCGGCCGCTTGGGTGG (50) 3'
PRIMER 2: 5' (277) AGCAGCCAGTCCCTTCCCGCTTCAG (253) 3'
Annealing temperature 50 °C

Ampicillin (bla) resistance gene of the Ti binary vector (using the pUC 19 numbering system described by Yannish-Perron et al., (1985).

PRIMER 1: 5' (1681) TCCATAGTTGCCTGACTCCCCG (1702) 3'
PRIMER 2: 5' (2000) TGGGAACCGGAGCTCAATGA (1981) 3'
Annealing temperature 60 °C

ros gene of Agrobacterium tumefacience chromosome (using the numbering system of Cooley et al., (1991), J. Bacteriology 173, 2608-2616); primers by Matzk and Schiemann (Poster No. S7-23, 8th International Congress of Plant Tissue and Cell Culture, Firenze, Italy, June 12-17 1994).

PRIMER 1: 5' (142) CGCGGGCTACAAGTTGAATC (161) 3'
PRIMER 2: 5' (714) GACCGAGACCCATTTCCTTG (695) 3'
Annealing temperature 60 °C

Vir G gene of the virulence gene of Agrobacterium tumefaciens Ti plasmid (using the numbering system of Chen et al., (1991), Mol Gen Genet 230, 302-309); primers designed by Matzk and Schiemann (Poster No. S7-23, 8th International Congress of Plant Tissue and Cell Culture, Firenze, Italy, June 12-17 1994).

PRIMER 1: 5' (370) GCCGACAGCACCCAGTTCAC (389)
PRIMER 2: 5' (749) GCCGTAAGTTTCACCTCACC (730)
Annealing temperature 60 °C

The band diagnostic for the NPT11 gene sequence acts as a reaction in that this product should be detected in both in the genetically modified plants and in plants infected with the Agrobacterium strain used for their production (EHA101A [pEHA101, pSCV1.6]). Presence of the band diagnostic for the presence of the bla gene is indicative of either transfer of additional DNA to the plant from the Ti binary vector due to incorrect processing at the left border region or of presence of Agrobacterium cells containing the binary Ti plasmid (pSCV1.6). Presence of the bands diagnostic for the ros and virG genes are indicative that the plants are still infected with the Agrobacterium strain used in their production (EHA101A [pEHA101, pSCV1.6]).

<u>Detection of contaminants in genetically manipulated plants using microbiological assay</u>

<u>Method</u>:

Genetically manipulated plants were homogenised in sterile mortar and pestles and the resultant homogenate was transferred aseptically to shake flasks containing sterile YEB medium (as descibed previously) without antibiotics. The flasks were incubated at 29 °C with vigorous shaking for a minimum of 5 days. Lack of microbial growth is indicative that the genetically manipulated plants are free of Agrobacterium.

<u>Results of PRC and microbiological analysis</u>

Using both the PCR method and the microbiological method, no Agrobacterium cells were detected in the genetically manipulated plants produced using the protocol described above.

REFERENCES

- Aitken-Christie et al (1994) In: Automation and environmental control in plant tissue culture, J Aitken-Christie; T Kozai & M A L Smith (Eds) Kluwer Academic Publishers

- Beck E; Ludwig G; Auerswald E A; Reiss B; Schaller H: (1982). Gene 19, 327-336.

- Draper, J., Scott, R., Armitage, P. and Walden, R. (1988). Plant genetic transformation and gene expression. A laboratory manual. Blackwell Scientific Publications, Oxford.

- Edwards G A. (1988): Ph. D. Thesis. University of Durham.

- Feinberg AP; Vogelstein B: (1983). Anal. Biochem. 132, p6-13.

- Fromm M; Taylor L P; Walbot V: (1986). Nature 319, 791-793.

- Guerineau, J.F. (1990). In: pJIT Plasmid Directory, Institute of Plant Science Research, John Innes Institute, Colney Lane, Norwich.

- George E F (1993/1996) Plant Propagation by Tissue Culture Volume 2, Exergetics Limited

- Heery, D.M., Gannon, F., and Powell, R. (1990). Trends Genet., 6, 173.

- Holsters B; Villaroel R; Gielen J; Seurinck J; De Greve H; Van Montagu M; Schell J: (1983). Mol. Gen. Genet. 190, 35-41.

- Hood E E; Helmer G L; Frayley R T; Chiton M-D: (1986). J. Bacteriol. 168, 1291-1301.

- Keil M; Griffin AR: (1994). Theor Appl Genet 89, 442-450.

- Murashige T. and Skoog F: (1962). Phys. Plant. 15, 473-497.

- Morel and Wetmore: (1951). Am. J Bot. 38, 141-143.

Olszewski N E; Martin F B; Ausubel F M: (1988). Nucleic

Acids Research 16, 10765-10782.

- Peralta E G; Hellmiss R; Ream W: (1986). EMBO J. 5, 1137- 1142.
- Sambrook J; Fritsch E F; Maniatis T: (1989). Molecular cloning: A laboratory manual. Second Edn. Cold Spring Harbour Press.
- Simon R; Priefer U; Puhler A: (1983). Biotechnology 1, 784-791.
- Simpson R B; O'Hara P J; Kwok W; Montoya A L; Lichenstein C; Gordon M P; Nester E W: (1982). Cell 29, 1005-1014.
- Thomas C M; Meyer R; Helsinki D R: (1980). J. Bacteriology 141, 213-222
- Valantine C R I; Kado C I: (1989). In: Promiscuous plasmids of the Gram-negative bacteria. C. M. Thomas (Ed). Academic Press. p125-163.
- Vancanneyt G; Schmidt R; O'Connor-Sanchez A; Willmitzer L; Rocha- Sosa M: (1990). Mol. Gen. Genet., 220, 245-250.
- Wen-jun S; Forde B G. (1989). Nucleic Acids Res. 17, 8385.
- Yannish-Perron C; Vieira J; Messing J: (1985). Gene 33, 103-119.
- Yenofsky R L; Fine M; Pellow J W: (1990). Proc. Natl Acad. Sci. USA. 87, 3435-3439.

CLAIMS

1. A process for the micropropagation of shoots, rooted shoots or seedlings of a woody plant, which comprises cultivating the shoots, rooted shoots or seedlings in an oxygenated liquid medium, the shoots, rooted shoots or seedlings being submerged in the liquid medium.

2. A process as claimed in claim 1, wherein oxygen is provided by passing air or oxygen through the medium, by mechanical agitation means, by means of shaking the medium, by means of illuminating the medium, or by any two or more of said means.

3. A process as claimed in claim 1, wherein the liquid medium is agitated.

4. A process as claimed in claim 3, wherein the culture medium is agitated and oxygen is provided by passing oxygen or air through the medium.

5. A process as claimed in any of one claims 1 to 4, wherein the shoots, rooted shoots or seedlings are free to move in the liquid medium.

6. A process as claimed in claim 5, wherein the shoots, rooted shoots or seedlings tumble in the liquid medium.

7. A process as claimed in any of one claims 1 to 4, wherein the movement of the shoots, rooted shoots or seedlings is restricted or otherwise impeded.

8. A process as claimed in claim 7, wherein the shoots, rooted shoots or seedlings are restrained in a perforated container within a vessel that contains the liquid medium or are restrained means within a section of the vessel that is separate from but in liquid contact with the liquid medium.

9. A process as claimed in any one of claims 1 to 8, wherein the shoots, rooted shoots or seedlings are

obtained from a cultivar, clone or seed.

10. A process as claimed in any one of claims 1 to 9, wherein the shoots, rooted shoots or seedlings are present at a high inoculation density.

11. A process as claimed in any one of claims 1 to 10, wherein the culture medium comprises an antibiotic.

12. A process as claimed in claim 11, wherein the shoots, rooted shoots or seedlings of the woody plant have been obtained from a plant grown under non-sterile conditions.

13. A process as claimed in claim 12, wherein the shoots, rooted shoots or seedlings have been obtained from genetically manipulated plant material transformed using an Agrobacterium-mediated transfer system.

14. A process as claimed in any one of claims 1 to 13, wherein the woody plant is a gymnosperm or dicotyledenous angiosperm used for wood pulp, for fuel or for timber; a tree, shrub or bush that produces fruit or nuts; a tree or shrub from which a commercially useful product other than a fruit or nut is obtained; or an ornamental tree or shrub.

15. A process as claimed in any one of claims 1 to 13, wherein the woody plant is of a sclerophyllous species.

16. A process as claimed in claim 15, where the woody plant is a Rhododendron, Azalea or Kalmia (Ericaceae); an Olea (Oleaceae); or an Australian Acacia.

17. A process as claimed in claim 14, wherein the woody plant is a Malus (apple); Pyrus, Prunus or Rosa (Rosaceae); Forsythia or Syringia (Oleaceae).

18. A process as claimed in claim 14, wherein the woody plant is a eucalypt.

19. A process as claimed in claim 18, wherein the woody plant is a eucalypt of the sub-genus Eucalyptus symphyomyrtus.

20. A process as claimed in claim 19, wherein the eucalypt is E. grandis, E. globulus, E. nitens, E.

dunnii, E. saligna, E. camaldulensis, E. urophylla or a hybrid thereof, or E. regnans, E. citriodora, E. fraxinoides or a hybrid thereof.

21. A process as claimed in any one of claims 1 to 20, wherein the cultivation is carried out in an air-lift fermenter.

22. A process as claimed in any one of claims 1 to 21, wherein cultivation of shoots is continued until rooting has initiated or has taken place.

23. A process as claimed in any one of claims 1 to 22, wherein a resulting shoot, rooted shoot or seedling is further micro-propagated by a process as claimed in any one of claims 1 to 22.

24. A process for selecting genetically modified shoots, rooted shoots or seedlings that have a selectable property, characteristic or attribute, wherein the shoots, rooted shoots or seedlings are cultivated submerged in an oxygenated liquid medium that comprises means for selecting the genetically manipulated shoots, rooted shoots or seedlings.

25. A process as claimed in claim 24, wherein the selectable property is resistance to an antibiotic, herbicide or other selective agent.

26. A process as claimed in claim 24 or claim 25, wherein oxygen is provided by passing air or oxygen through the medium, by mechanical agitation means, by means of shaking the medium, by means of illuminating the medium, or by any two or more of said means.

27. A process as claimed in claim 24 or claim 25, wherein the liquid medium is agitated.

28. A process as claimed in claim 24, wherein the culture medium is agitated and oxygen is provided by passing oxygen or air through the medium.

29. A process as claimed in any of one claims 24 to 28, wherein the shoots, rooted shoots or seedlings are free

to move in the liquid medium.

30. A process as claimed in claim 29, wherein the shoots, rooted shoots or seedlings tumble in the liquid medium.

31. A process as claimed in any of one claims 24 to 28, wherein the movement of the shoots, rooted shoots or seedlings is restricted or otherwise impeded.

32. A process as claimed in claim 31, wherein the shoots, rooted shoots or seedlings are restrained in a perforated container within a vessel that contains the liquid medium or are restrained within a section of the vessel that is separate from but in liquid contact with the liquid medium.

33. A process as claimed in any one of claims 24 to 32, wherein the shoots, rooted shoots or seedlings are obtained from a cultivar, clone or seed.

34. A process as claimed in any one of claims 24 to 33, wherein the shoots, rooted shoots or seedlings are present at a high inoculation density.

35. A process as claimed in any one of claims 24 to 34, wherein the culture medium comprises an antibiotic.

36. A process as claimed in claim 35, wherein the shoots, rooted shoots or seedlings of the woody plant have been obtained from a plant grown under non-sterile conditions.

37. A process as claimed in claim 36, wherein the shoots, rooted shoots or seedlings have been obtained from genetically manipulated plant material transformed using an Agrobacterium-mediated transfer system.

38. A process as claimed in claim 37, wherein shoot formation is induced in the Agrobacterium-transformed cells or tissue in the presence of N-(2-chloro-4-pyridyl)-N'-phenylurea or another phenylurea.

39. A process as claimed in any one of claims 24 to 38, wherein the genetically modified shoots, rooted shoots or seedlings are from an annual, biennial or perennial plant.

40. A process as claimed in any one of claims 24 to 39, where the genetically modified shoots, rooted shoots or seedlings are from a monocotyledenous or dicotyledenous plant.

41. A process as claimed in any one of claims 24 to 40, where the genetically modified shoots, rooted shoots or seedlings are from a herbaceous plant.

42. A process as claimed in any one of claims 24 to 40, wherein the shoots, rooted shoots or seedlings are of a woody plant.

43. A process as claimed in claim 42, wherein the woody plant is a gymnosperm or dicotyledenous angiosperm used for wood pulp, for fuel or for timber; a tree, shrub or bush that produces fruit or nuts; a tree or shrub from which a commercially useful product other than a fruit or nut is obtained; or an ornamental tree or shrub.

44. A process as claimed in claim 42, wherein the woody plant is of a sclerophyllous species.

45. A process as claimed in claim 44, where the woody plant is a Rhododendron, Azalea or Kalmia (Ericaceae); an Olea (Oleaceae); or an Australian Acacia.

46. A process as claimed in claim 42, wherein the woody plant is a Malus (apple); Pyrus, Prunus or Rosa (Rosaceae); Forsythia or Syringia (Oleaceae).

47. A process as claimed in claim 42, wherein the woody plant is a eucalypt.

48. A process as claimed in claim 47, wherein the woody plant is a eucalypt of the sub-genus Eucalyptus symphyomyrtus.

49. A process as claimed in claim 48, wherein the eucalypt is E. grandis, E. globulus, E. nitens, E. dunnii, E. saligna, E. camaldulensis, E. urophylla or a hybrid thereof, or E. regnans, E. citriodora, E. fraxinoides or a hybrid thereof.

50. A process as claimed in any one of claims 24 to 41,

wherein the plant is a non-woody plant.

51. A process as claimed in any one of claims 24 to 50, wherein the cultivation is carried out in an air-lift fermenter.

52. A process for the production of genetically modified shoots, which comprises subjecting cells or tissue of a plant to Agrobacterium-mediated transfer of one or more DNA sequence(s) of interest, inducing shoot formation in the resulting transformed cells or tissue, which have a selectable characteristic, property or attribute, in the presence of an agent capable of inducing the fomration of shoots and selecting the resulting shoots in an oxygenated liquid culture medium that comprises means for selecting the genetically modified shoots, the shoots being submerged in the liquid medium.

53. A process as claimed in claim 52, wherein the shoot inducing agent is a cytokinin.

54. A process as claimed in claim 53, wherein the shoot inducing agent in BAP, N-(2-chloro-4-pyridyl)-N'-phenylurea or another phenylurea.

55. A process as claimed in any one of claims 52 to 54, wherein the shoot inducing agent is also present in the liquid culture medium.

56. A process as claimed in any one of claims 52 to 55, carried out under as defined in any one of claims 24 to 51.

57. A process as claimed in any one of claims 24 to 56, wherein the selection in the liquid culture medium is carried out before and/or after selection on a solid (gelled) medium that comprises means for selecting the genetically modified shoots, rooted shoots or seedlings.

58. A process for the micropropagation of a genetically modified shoot, rooted shoot or seedling, wherein a a genetically modified shoot, rooted shoot or seedling selected or produced according to a process as claimed in

any one of claims 24 to 57 is micropropagated according to a process as claimed in any one of claims 1 to 23.

59. A process for reducing microbial contamination of shoots, rooted shoots or seedlings, which comprises cultivating the shoots, rooted shoots or seedlings in an oxygenated liquid medium that comprises an antibiotic, the shoots, rooted shoots or seedlings being submerged in the liquid medium.

60. A process as claimed in claim 59 carried out as defined in any one of claims 1 to 13.

61. A process as claimed in claim 59 or claim 60, wherein the shoots, rooted shoots or seedlings are of a plant as defined in any one of claims 39 to 50.

62. Use of cultivation of shoots, rooted shoots or seedlings in an oxygenated liquid medium that comprises an antibiotic, the shoots, rooted shoots or seedlings being submerged in the liquid medium, for the reduction of microbial contamination of the shoots, rooted shoots or seedlings.

63. A plant obtained from a shoot, rooted shoot or seedling obtained by a process as claimed in any one of claims 1 to 22 or from a genetically modified shoot, rooted shoot or seedling selected according to a process as claimed in any one of claims 24 to 58 or from a shoot, rooted shoot or seedling having reduced microbial contamination obtained according to a process as claimed in any one of claims 59 to 62.

64. A process as claimed in any one of claims 1 to 23, wherein the shoots, rooted shoots or seedlings are obtained from a plant as claimed in claim 63.

65. A process as claimed in claim 64, wherein the plant is a mature eucalypt.

66. A product obtained from a plant as claimed in claim 63.

FIG. 1

3b

3a

2

1

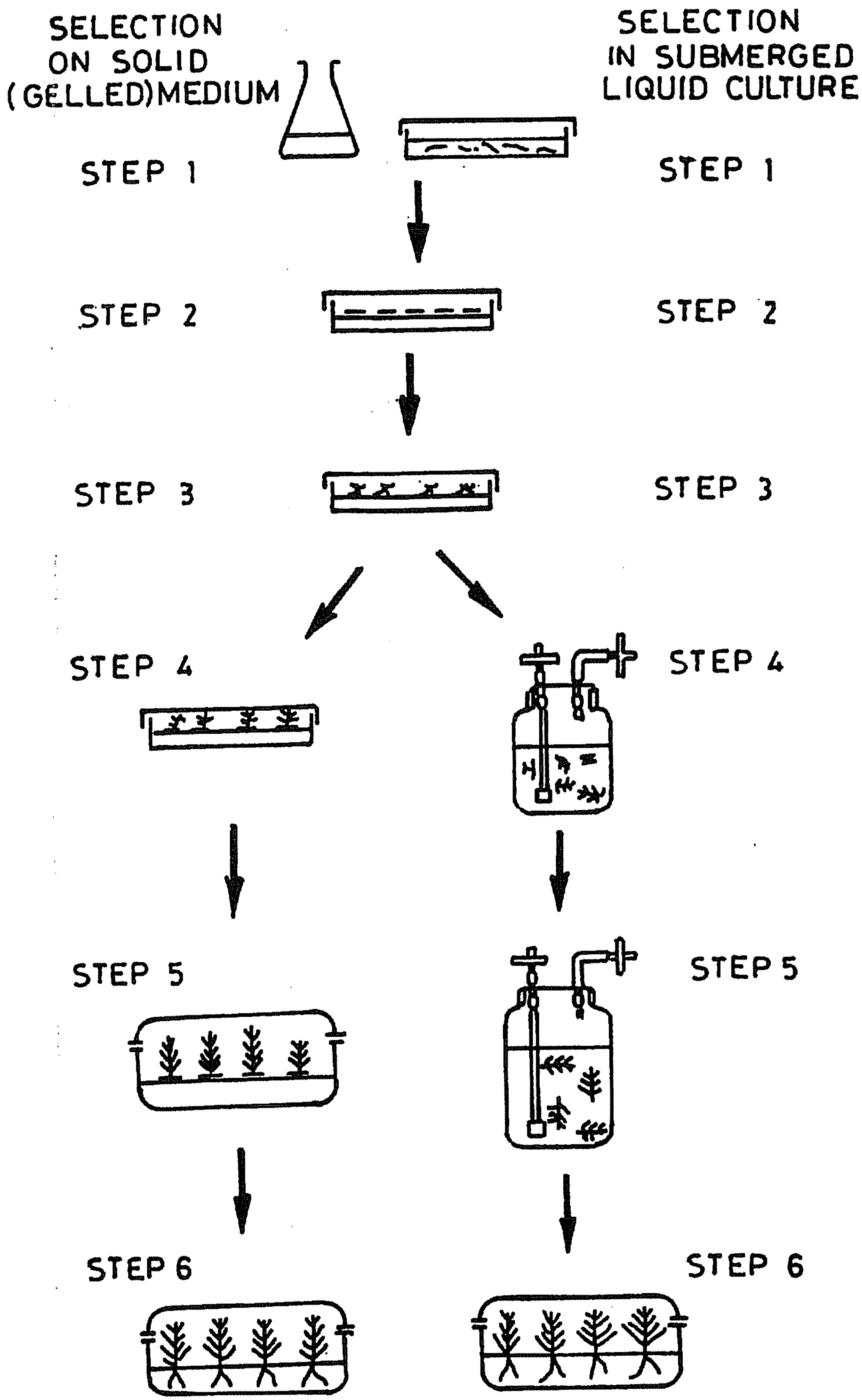

SELECTION
ON SOLID
(GELLED)MEDIUM
SELECTION
IN SUBMERGED
LIQUID CULTURE
STEP 1
STEP 1
STEP 2
STEP 2
STEP 3
STEP 3
STEP 4
STEP 4
STEP 5
STEP 5
STEP 6
STEP 6

FIG.2

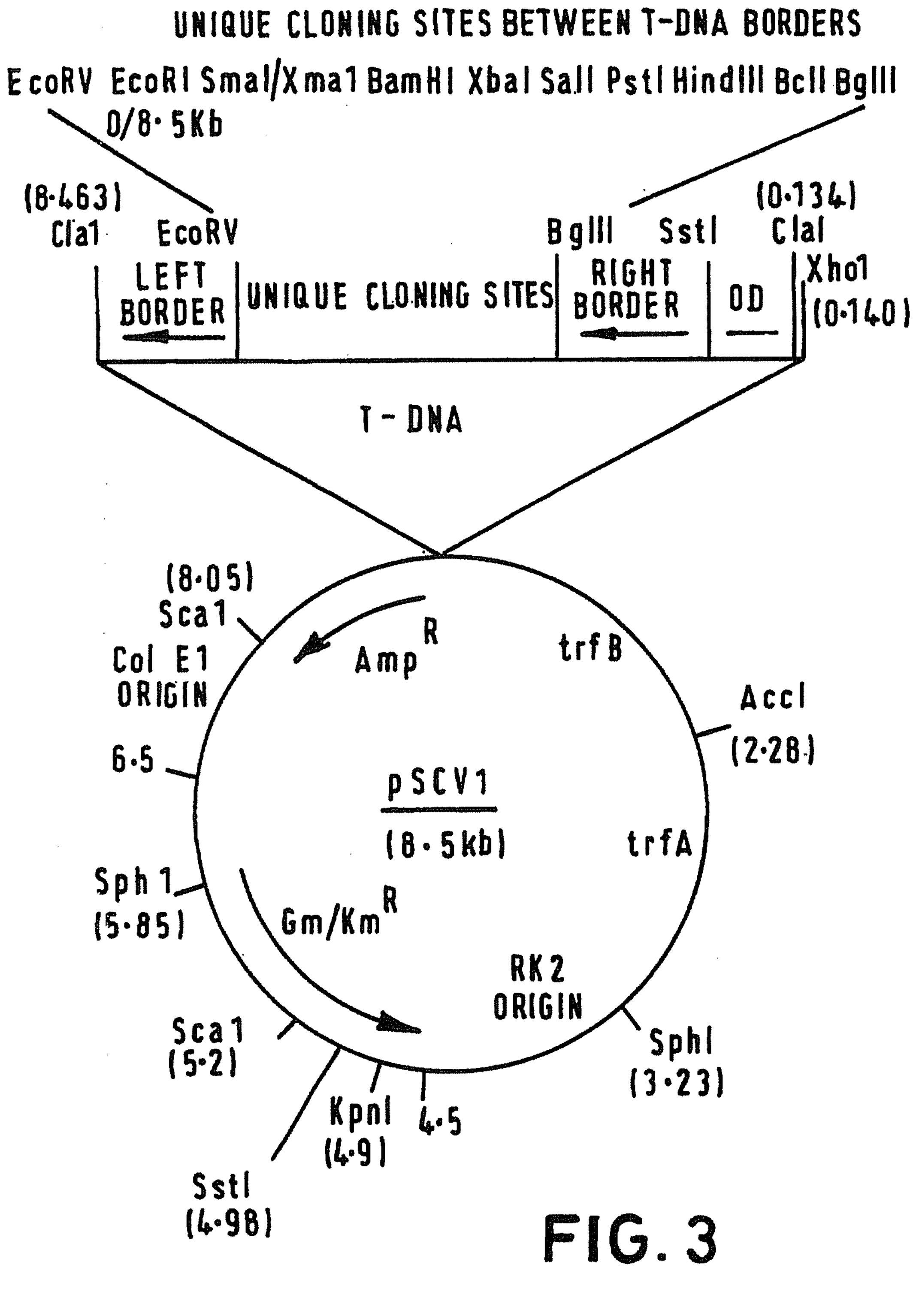

UNIQUE CLONING SITES BETWEEN T-DNA BORDERS
EcoRV EcoRI SmaI/Xma1 BamHI XbaI SalI PstI HindIII BclI BglII
0/8·5Kb
(8·463)
Cla1
EcoRV
BglII
SstI
(0·134)
ClaI
XhoI
(0·140)
LEFT BORDER
UNIQUE CLONING SITES
RIGHT BORDER
OD
T-DNA
(8·05)
Sca1
Col E1 ORIGIN
Amp^R
trfB
AccI
(2·28)
6·5
pSCV1
(8·5kb)
trfA
Sph 1
(5·85)
Gm/Km^R
RK2 ORIGIN
Sca1
(5·2)
SphI
(3·23)
KpnI
(4·9)
4·5
SstI
(4·98)

FIG. 3

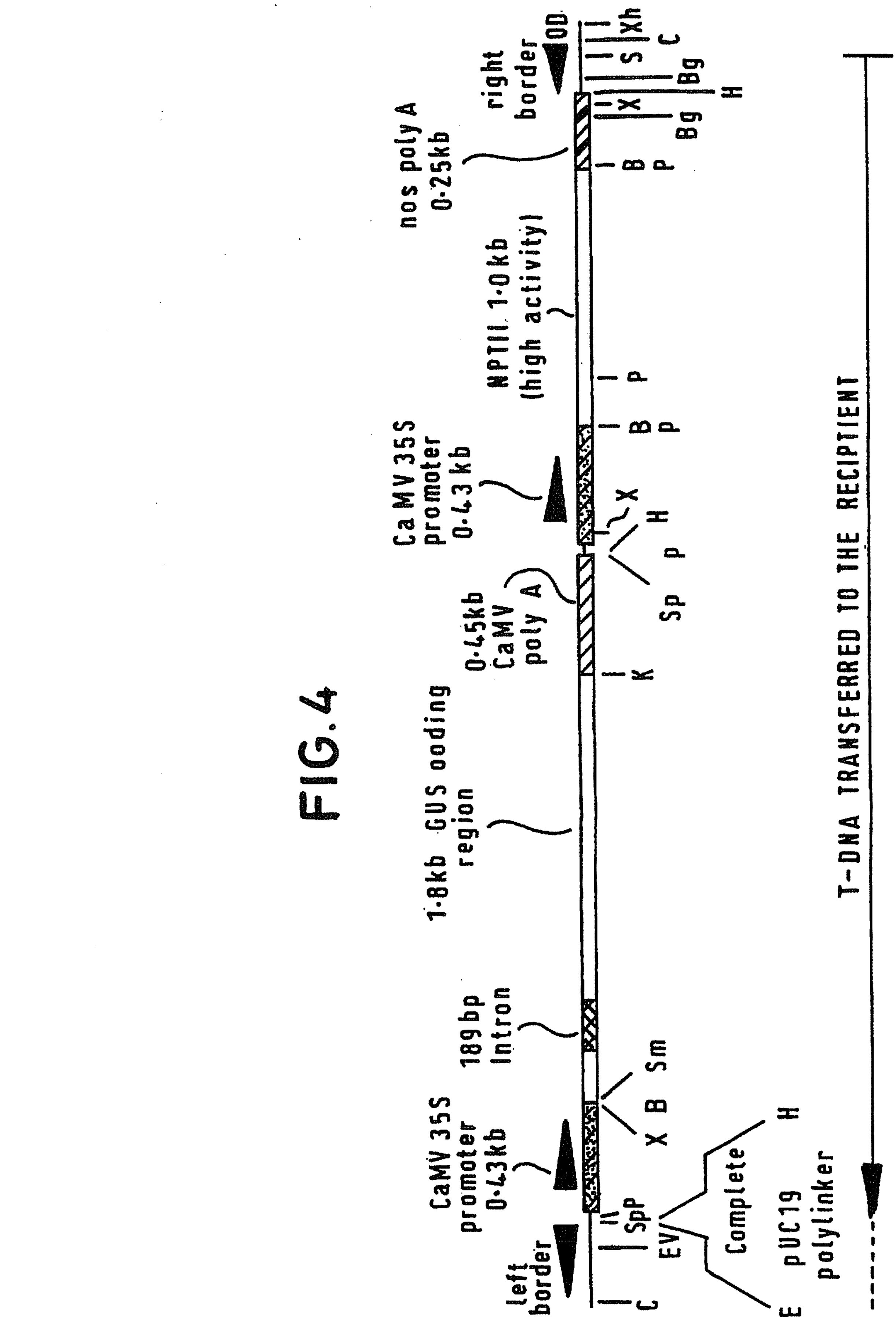

left border
CaMV 35S promoter 0·43kb
189bp Intron
1·8kb GUS ooding region
0·45kb CaMV poly A
CaMV 35S promoter 0·43kb
NPTII 1·0kb (high activity)
nos poly A 0·25kb
right border
C
Sp P
EV
E
X B Sm
H
Complete pUC19 polylinker
K
Sp P
H
X
B P
P
B P
Bg
X
Bg
H
S
C
Xh
OD
T-DNA TRANSFERRED TO THE RECIPTIENT

FIG. 4